UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

MARE ENDOMETRIUM- PHYSIOLOGICAL AND PATHOLOGICAL INVOLVEMENT OF HORMONES AND NEUTROPHIL EXTRACELLULAR TRAPS

Maria Rosa Rebordão Cordeiro Simões Crisóstomo

Orientador

Doutora Graça Maria Leitão Ferreira Dias

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Ciências Biológicas e Biomédicas

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To João, Mafas and Jota

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THESIS TITLE: Mare endometrium- physiologic and pathologic involvement of hormones and neutrophil extracellular traps

ABSTRACT

Two reproductive topics in mares were addressed in this thesis. The aims of the studies were to evaluate: (i) the effect of chronic oxytocin administration to mid-luteal phase mares on luteal maintenance and its cellular and molecular mechanisms at endometrial level; (ii) the capacity of equine neutrophils to produce neutrophil extracellular traps (NETs) *in vitro* when stimulated with bacteria obtained from mares with endometritis, and to determine if NETs release also occurred *in vivo* in mares with endometritis; (iii) the *in vitro* effects of some NETs components on mare endometrial fibrogenic capacity and to determine if they could depend on endometrial inflammatory lesions or estrous cycle phases; and (iv) the involvement of PGF2α and PGE2 pathways in collagen deposition on mare endometrium, challenged with NETs proteases. In the first study, luteal maintenance occurred in 67% of oxytocin treated mares, which may be related to oxytocin and progesterone (PGR) receptors spatial expression in endometrium. Reduction of endometrial estrogen receptor 2 (ESR2) may be responsible for the maintenance of PGR in luminal and glandular epithelium and may attenuate *ESR1* endometrial transcriptional activity. Equine neutrophils were able to release NETs in the presence of bacteria that cause mare endometritis, and might be a complementary mechanism to fight endometritis. By *in vitro* studies with NETs proteases, increased collagen type I (COL1) production characteristic of fibrosis was observed, although endometrial response to each NETs protease depended on estrous cycle and/or endometrial category. Also, NETs proteases were linked to fibrogenesis, by increased synthesis of PGF2α and/or PGF2α receptor transcripts and impaired PGE2 or PGE2 receptor 2 transcripts associated to increased COL1. These effects were influenced by endometrium type and estrous cycle phases. Injury induced-changes on PG mediators by NETs components may instigate PGF2α or PGE2 vias, as additional pathways in mare endometrial fibrogenesis.

Key words

Oxytocin; neutrophil extracellular traps (NETs), mare, endometrial fibrosis, prostaglandins

Título da dissertação: Endométrio da égua: envolvimento fisiológico e patológico de hormonas e de redes extracelulares de neutrófilos.

Resumo:

Nesta tese foram abordados dois temas reprodutivos em éguas. Os objectivos destes estudos consistiram na avaliação: (i) da administração crónica de ocitocina na manutenção do corpo lúteo em éguas na fase lútea média, e os mecanismos celulares e moleculares no endométrio; (ii) da capacidade dos neutrófilos equinos para produzirem redes extracelulares de neutrófilos (NETs) *in vitro* e *in vivo* quando estimulados com bactérias de éguas com endometrite; (iii) dos efeitos *in vitro* de componentes das NETs na fibrogénese do endométrio equino e sua associação com lesões inflamatórias no endométrio ou com o ciclo éstrico; e (iv) do envolvimento das vias da PGF2α e PGE2 na deposição de colagénio no endométrio da égua, incubado com proteases das NETs. No primeiro estudo, o prolongamento da função lútea em 67% das éguas tratadas com ocitocina, parece estar relacionado com alteração da expressão espacial dos receptores de ocitocina e progesterona (PGR) no endométrio. A diminuição do receptor 2 dos estrogénios (ESR2) pode ser responsável pela manutenção do PGR no epitélio luminal e glandular do endométrio e atenuar a transcrição do ESR1. Os neutrófilos equinos libertaram NETs na presença de bactérias causadoras de endometrite podendo ser um mecanismo complementar no combate à endometrite. As proteases das NETs *in vitro* aumentaram a produção de colagénio do tipo I (COL1), característico de fibrose, no endométrio, embora a resposta a cada protease dependesse do ciclo éstrico e/ou da categoria do endométrio. O aumento do COL1 associado ao incremento de PGF2α e/ou da transcrição do seu receptor e à diminuição da PGE2 e/ou do seu receptor 2, parecem implicar as prostaglandinas e as proteases das NETs na fibrogénese no endométrio equino. Alterações nos mediadores das prostaglandinas, induzidas pelos componentes das NETs, podem instigar as vias da PGF2α ou da PGE2, como mecanismos adicionais de fibrose do endométrio da égua.

Palavras-chave

Oxitocina; redes extracelulares dos neutrófilos (NETs); égua; fibrose do endométrio; prostaglandinas

Título da dissertação: Endométrio da égua: envolvimento fisiológico e patológico de hormonas e de redes extracelulares de neutrófilos.

Resumo alargado:

Nos cavalos envolvidos em actividades desportivas o comportamento do estro está associado a um efeito negativo no desempenho e no treino, devido a uma mudança na atitude da égua, que se pode expressar por comportamentos distintos. Dentro dos vários métodos possíveis de supressão do estro, a administração exógena de ocitocina, nos dias 7 a 14 do ciclo éstrico, demonstrou ser um método eficaz de supressão de estro a longo prazo em éguas de desporto.
A endometrose, que consiste no processo degenerativo crónico do endométrio, tem sido uma grande preocupação no campo reprodutivo da égua levando à morte embrionária precoce. Parece existir uma relação entre o grau de fibrose do endométrio e a taxa de fecundidade. Embora seja um dos principais temas de investigação na área reprodutiva da égua, a etiologia e os mecanismos que levam à fibrose do endométrio continuam por elucidar. Na égua, a endometrite persistente devido à presença de bactérias ou sémen no útero induz um estado inflamatório caracterizado por um influxo de neutrófilos para o lúmen uterino. Esta situação quando continuada, pode originar o desenvolvimento de uma inflamação crónica, com a activação de células fibróticas e alterações degenerativas crónicas no endométrio, relacionadas com a formação de fibrose. No entanto, a associação entre o desenvolvimento da endometrose e a inflamação crónica do endométrio continua a ser um tópico controverso.
Estes dois temas atrás mencionados, relativos à reprodução em éguas, foram o foco principal deste trabalho. No primeiro estudo avaliou-se o efeito da administração crónica de ocitocina exógena na manutenção da função lútea em éguas. Foi igualmente estudada a expressão no endométrio equino das sintases da prostaglandina endoperoxidase 2 (PTGS2), $F_2\alpha$, E_2 e I_2 (PTGFS e PTGES) e dos receptores de ocitocina (OXTR), progesterona (PGR) e estradiol (ESR1 e ESR2). No ensaio *in vivo*, foi administrada ocitocina a éguas entre os dias 7 e 14 do ciclo éstrico. Como se pretendeu comparar as condições do endométrio da fase lútea média antes da luteólise com as condições após a supressão da luteólise induzida pela administração de OXT, os estudos de expressão no endométrio foram realizados em biopsias obtidas no dia 10 pós-ovulação nas éguas controlo e no dia 24 em éguas com função lútea persistente. A administração de OXT às éguas na fase lútea média foi eficaz no bloqueio da luteólise e prolongou a fase lútea em 67% (4/6) das éguas tratadas com OXT. A função lútea prolongada associada ao tratamento crónico com OXT parece estar relacionada com um aumento e com uma diferente expressão espacial dos OXTR e PGR no endométrio. Nas éguas tratadas com OXT, foi detectado um aumento da expressão dos OXTR nas células do estroma do

endométrio. Uma vez que durante os eventos fisiológicos que ocorrem na fase lútea tardia do ciclo éstrico é necessário um aumento da expressão dos OXTR no epitélio superficial do endométrio para desencadear a luteólise, a administração crónica de OXT parece desviar a expressão dos OXTR do epitélio do lúmen uterino para o estroma, levando deste modo à manutenção da função lútea. Além disso, a diminuição da expressão dos ESR2 observada nas éguas tratadas com OXT pode ser responsável pela manutenção dos PGR no epitélio superficial e glandular do endométrio.

O segundo estudo, teve como objectivo avaliar a capacidade dos neutrófilos equinos para produzir NETs não só *in vitro* após estimulação com estirpes *Streptococcus equi* spp *zooepidemicus*, *Escherichia coli* ou *Staphylococcus capitis* obtidas de endometrite das éguas, mas também *in vivo* nas secreções do endométrio de éguas com endometrite bacteriana. Neste estudo, conseguimos mostrar que os PMN equinos estimulados são capazes de libertar NETs na presença de bactérias Gram-positivas e Gram-negativas específicas que causam infecção uterina na égua. Além disso, este estudo mostrou que as bactérias que causavam endometrite na égua estavam presas nas NETs mostrando existir um contacto directo entre as NETs e as bactérias. Além disso, a expressão de histonas, mieloperoxidase e elastase nas NETs *in vivo,* sugere que as NETs possam ter um efeito antimicrobiano, actuando como um possível mecanismo complementar pelo qual as éguas são capazes de resistir à endometrite.

Na terceira parte deste trabalho, postulámos que a persistência dos componentes das NETs pode desencadear um efeito nocivo no endométrio, levando à deposição de colagénio e ao desenvolvimento de fibrose na égua. Através de estudos *in vitro* em que explantes dos endométrios foram incubados com diferentes doses de proteases das NETs (elastase, mieloperoxidase e catepsina), abordámos a persistência de NETs como potenciais mediadores de fibrose no endométrio em éguas susceptíveis à endometrite. A influência da fase do ciclo éstrico, bem como a ausência ou a presença de alterações patológicas no endométrio foram também avaliadas. Neste estudo, observou-se no endométrio um aumento da produção de COL1, característico de fibrose, quando na presença de todas as proteínas das NETs estudadas, embora a resposta do endométrio a cada protease específica dependesse da fase do ciclo éstrico e/ou da categoria do endométrio. No entanto, a elastase aumentou a produção de COL1 em todas as categorias de endométrios, independentemente da fase do ciclo éstrico. Além disso, todas as proteases das NETs estudadas induziram um aumento da produção de COL1 nos endométrios obtidos na fase folicular, independentemente da presença de alterações patológicas. Assim, a fase folicular parece ser a fase do ciclo éstrico em que a égua apresenta maior risco de desenvolver essa patologia.

Nas duas últimas partes deste trabalho, foi abordado um dos múltiplos possíveis mecanismos envolvidos na patogénese extremamente complexa da fibrose do endométrio da égua. Também fazendo uso de estudos *in vitro* com as proteases das NETs, as vias de prostaglandinas foram consideradas como potenciais mediadores do desenvolvimento de fibrose do endométrio devido à persistência das NETs. No nosso estudo, o aumento da síntese de PGF2α após 24h de incubação de endométrio equino com elastase ou catepsina só foi detectado em endométrios obtidos na fase folicular e sem alterações anatomopatológicas ou com ligeiras lesões patológicas e associado a um aumento da produção do COL1. No entanto, o aumento da produção de COL1 e da transcrição do gene do receptor da PGF2α (*FP*) foram observados em todas as categorias de endométrios obtidos na fase folicular, após o tratamento com catepsina. Também foi notado um aumento dos níveis de transcrição dos *FP* em endométrios com lesões moderadas a intensas, obtidos na fase lútea média. Ambas as circunstâncias podem estimular o desenvolvimento de fibrose no endométrio da égua, uma vez que na espécie humana, os aumentos da síntese de PGF2α actuando no seu próprio receptor (FP) estão relacionados com a formação de fibrose em vários sistemas orgânicos.

A PGE2, ao sinalizar através dos seus receptores 2 (EP2) e 4 (EP4), é considerada um mediador anti-fibrótico. Ao diminuírem os níveis de PGE2 ou do EP2 nos tecidos, a PGE2 deixa de poder exercer as suas funções anti-fibróticas. Os nossos resultados sugerem que no endométrio da égua, o *EP2* possa ser responsável pelos possíveis efeitos anti-fibróticos da PGE2. No presente estudo, foi observada uma diminuição da síntese de PGE2 no endométrio de éguas com lesões moderadas a intensas, independentemente da fase do ciclo éstrico. Uma vez que o aumento da produção de COL1 e a diminuição dos transcritos de *EP2* só foram observados em endométrios obtidos na fase folicular após incubação com elastase, sugere que a diminuição do *EP2* possa estar envolvida no mecanismo de fibrogénese neste tipo de endométrio. Assim, em condições fisiológicas, os endométrios na fase folicular parecem ser mais propensos a desenvolver fibrose na presença de um estímulo pró-fibrótico, devido a uma menor capacidade para sintetizar o mediador anti-fibrótico *EP2*. Além disso, em endométrios saudáveis ou com ligeiras alterações patológicas obtidos na fase lútea média, a via da PGE2 pode desempenhar um papel chave no desencadeamento de fibrose, devido a uma supressão da acção anti-fibrótica da *PGE2*. O aumento da produção de COL1 induzido pela elastase e catepsina nestes tipos de endométrios estava associado com uma diminuição da síntese de PGE2 ao longo do período experimental, sem alterações nos outros mediadores da via de PGE2 estudados e ligado a baixos níveis de expressão do gene *FP*. Portanto, os nossos resultados sugerem que alterações nos mediadores das prostaglandinas, induzidas pelos componentes das NETs, podem fomentar as vias da PGF2α ou da PGE2, como mecanismos

adicionais de fibrose do endométrio da égua. No entanto, embora os nossos dados *in vitro* sugiram o envolvimento das NETs no desenvolvimento da endometrite crónica na égua, podendo actuar como possíveis mediadores de fibrose do endométrio, são necessários mais estudos para confirmar a sua acção em condições *in vivo*.

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AA- arachidonic acid
ACE- angiotensin-converting enzyme
ADSC- adipose-derived stem cell
AKR1C3- aldo-keto reductase family 1 member C3
Akt- serine-threonine protein kinase
ALK- activin like kinase receptors
ANOVA- analysis of variance
B2M- beta-2-microglobulin
BM-MSC- bone marrow mesenchymal stem cell
BMP- bone morphogenetic protein
CAT- cathepsin G
CL- *corpus luteum*
CLR- C- type lectin receptors
COL1- type I collagen
COL3- type III collagen
CONT- control
Cq- cyclic threshold
CTGF- connective tissue growth factor
CV- coefficient of variation
CXCL8- C-X-C motif chemokine ligand 8
Cyt- cytochalasin
DAMP- damage-associated molecular patterns
DNA- deoxyribonucleic acid
E2- estrogens
eAT-MSCs- adipose tissue-derived multipotent mesenchymal stem cells
ECM- extracellular matrix
Ecoli- *Escherichia coli*
EGF- epidermal growth factor
EIA- enzyme immunoassay
ELA- elastase
EMT- epithelial-mesenchymal transition
EP- prostaglandin E2 receptors
EPF- endometrial periglandular fibrosis
ESR- estrogen receptors
FLP- follicular phase
FP- prostaglandin F2α receptor
GAPDH- glyceraldehyde 3-phosphate dehydrogenase
GE- glandular epithelia
GM-CSF- granulocyte-macrophage colony-stimulating factor
GPCR- G protein-coupled receptors
hCG- human chorionic gonadotropin
HSC- hepatic stellate cell
ICAM- intracellular adhesion molecule
IFN- interferon
IHC- immunohistochemistry
IL- interleukin
im- intramuscular
IPF- idiopathic pulmonary fibrosis
IUA- intrauterine adhesions
IUD- intrauterine devices

iv- intravenous
LE- luminal and epithelia
LPS- lipopolysaccharide
LTB4- leukotriene B4
M1- M1 macrophages
M2- M2 macrophages
MAPK- mitogen activated protein kinase
MLP- mid-luteal phase
MMP- matrix metalloproteinase
mPTGES- microsomal prostaglandin E synthase
MPO- myeloperoxidase
mRNA- messenger ribonucleic acid
MSC- Mesenchymal stem cell
MT-MMP- membrane type matrix metalloproteinase
NADPH- nicotinamide adenine dinucleotide phosphate
NE- neutrophil-derived elastase
NETs- neutrophil extracellular traps
NFκB- nuclear factor kappa B
NK- natural killer cells
NLR- Nod-like receptor
OXT- oxytocin
OXTR- oxytocin receptor
P4- progesterone
PAD4- protein-arginine deiminase 4
PAMP- pathogen-associated molecular pattern
PDGF- platelet-derived growth factor
PDGFR- platelet-derived growth factor tyrosine kinase receptor
PG- prostaglandin
PGD2- prostaglandin D2
PGE2- prostaglandin E2
PTGES- prostaglandin E2 synthase
PGF2a- prostaglandin F2a
PGG2- prostaglandin G2
PGH2- prostaglandin H2
PGI2- prostaglandin I2
PGR- progesterone receptor
PI3K- phosphatidyl inositol 3 kinase
PKA- protein kinase A
PKC- protein kinase C
PLA2- phospholipase A2
PMA- phorbol-myristate acetate
PMN- polymorphonuclear neutrophil
PRR- pattern recognition receptor
PTC- proximal tubular cells
PTGIS- prostaglandin I2 synthase
PTGS- prostaglandin-endoperoxide synthase
qPCR- real-time polymerase chain reaction
RLR- LIG-I-like receptors
ROS- reactive oxygen species
RPL32- ribosomal protein L32
S.D.- standard deviation
Scap- *Staphylococcus capitis*

SDHA- succinate dehydrogenase complex flavoprotein subunit A
SEM-standard error of the mean
SMA- smooth muscle actin
Smad- intracellular proteins that transduce extracellular signals from TGF-β ligands
SS- systemic sclerosis
ST- standard protein
Szoo- *Streptococcus equi subspecies zooepidemicus*
TGF- transforming growth factor
TGFBR- transforming growth factor-β receptor
Th- T-helper cell
TIMP- tissue inhibitor of metalloproteinase
TLR- Toll-like receptors
TNF- tumor necrosis factor
VEGF- vascular endothelial growth factor

Chapter I- *General introduction and objectives*

1. Introduction

In equine sports avoiding estrus behavior is a major concern, since it is associated with a negative effect in the performance or training of horses, due to a change in mare attitude, which can be expressed by distinct behaviors (Vanderwall, 2013). In the reproductive management of mares to control estrus behavior, it is important to take into consideration the effects of those practices on mare reproductive efficiency not only during their athletic careers but also on their future reproductive function. Different methods have been used to suppress estrus in the mare. Between them, exogenous administration of oxytocin (OXT) has been proved to be an effective and reproductively-safe long-term method of suppressing estrus in performance mares (Vanderwall *et al*., 2016). This hormone has dual effects depending on the endometrial endocrine environmental and when used exogenously can act in a dissimilar way and alter reproductive physiological events (Rebordão *et al*., 2016). Although OXT is essential to trigger luteolysis during the physiological events that occur in the late luteal phase of mare estrous cycle, it also acts as anti-luteolytic agent, inducing luteal maintenance when it is exogenously administrated from day 7 to 14 of the estrous cycle (Vanderwall *et al*., 2016; Rebordão *et al*., 2017a).

Mare endometrosis has been a major concern in mare reproductive field leading to early embryonic death and a direct link between the degree of fibrosis and the foaling rate has been proposed (Kenney & Doig, 1986). This source of subfertility/infertility is responsible for major economic losses in horse industry, since performance mares are used in sport activities until an old age when endometrial fibrosis is frequently present. In the mare, persistent endometritis due to different insults like microorganisms or semen involves the influx of large numbers of polymorphonuclear neutrophils (PMN) and other proteins into the uterine lumen, which may lead to chronic inflammation with activated fibrotic stromal cells and chronic degenerative changes of mare endometrium and related with fibrosis formation (LeBlanc & Causey, 2009; Hoffmann *et al*., 2009; Cadario, 2014). PMN are able to release neutrophil extracellular traps (NETs) when stimulated by different stimuli, like bacteria and semen (Brinkmann *et al*., 2004; Alghamdi & Foster, 2005). Although nucleic and cytoplasmic proteins and DNA associated proteins of NETs possess strong antimicrobial properties their persistence may cause disease themselves (Brinkmann & Zychlinsky, 2012).

These two issues regarding reproduction in mares were the main focus of this work. In the first part of this study, we investigate cellular and molecular mechanisms behind the exogenous use of oxytocin to alter the normal physiological luteolysis and prolong luteal function. In the second part, we prove that equine PMN are able to release NETs in the

presence of bacteria that cause mare endometritis, not only *in vitro*, but also *in vivo*, demonstrating a possible complementary mechanism by which mares can resist endometritis. In the third part, by *in vitro* studies with NETs proteases, we address persistence of NETs as potential mediators of mare endometrial fibrosis in mares susceptible to endometritis. The influence of estrous cycle phase and healthy or pathological conditions of endometrial tissues are also studied. In the last part of this work, also making use of *in vitro* studies with NETs proteases, we attempt to unravel prostaglandins pathways as putative mediators that trigger the development of endometrial fibrosis due to the presence of NETs persistence. By understanding the mechanisms involved in luteostasis and in endometrial fibrosis development in the mare, this work will contribute not only for advancing scientific knowledge but also to improve reproductive efficiency in horses and to reduce economic losses in the horse industry.

2. Objectives

The major objectives of this work were:

1)- To investigate the effect of chronic oxytocin administration to mares in mid-luteal phase to prolong luteal function and if that effect may be achieved by modulation of PG pathways, steroid hormones and OXT receptors in the endometrium.
The developed work was published: Rebordão, M.R., Galvão, A., Pinto-Bravo, P., Pinheiro, J., Gamboa, S., Silva, E., Mateus, L. & Ferreira-Dias, G. (2017a). Expression of prostaglandin synthases, ovarian steroids and oxytocin receptors in equine endometrium during luteal maintenance induced by chronic administration of oxytocin. *Theriogenology*, 87, 193–204.

2)- To evaluate the *in vitro* capacity of equine PMN to secrete NETs by chemical activation, or when stimulated with bacteria strains obtained from mares with endometritis, and to determine if NETs release also occurred *in vivo* in the endometrium of mares with bacterial endometritis.
The developed work was published: Rebordão, M.R., Carneiro, C., Alexandre-Pires, G., Brito, P., Pereira, C., Nunes, T., Leitão, A., Vilela, C. & Ferreira-Dias, G. (2014). Neutrophil extracellular traps formation by bacteria causing endometritis in the mare. *J Reprod Immunol*, 106, 41-49.

3) To assess the *in vitro* effect of some NETs proteases on mare endometrial fibrogenic capacity and determine if that effect could depend on endometrial inflammatory lesions or estrous cycle phase.
The developed work was submitted for publication: Rebordão, M.R., Amaral, A., Lukasik, K., Szóstek-Mioduchowska, A., Pinto-Bravo, P., Galvão, A., Skarzynski, D.J. & Ferreira-Dias, G. (2018). Constituents of neutrophil extracellular traps induce *in vitro* collagen formation in mare endometrium. *Theriogenology* (THE 14442; in press).

4) To investigate the role of PGF2α pathway on the potential ability of NETs components to induce endometrial fibrosis and if that effect could be influenced by endometrial inflammatory/fibrotic lesions or estrous cycle phase.
The developed work will be submitted for publication: Rebordão, M.R., Amaral, A., Lukasik, K., Szóstek-Mioduchowska, A., Pinto-Bravo, P., Galvão, A., Skarzynski, D.J. & Ferreira-Dias,

G. (2017). Neutrophil extracellular traps proteases stimulate fibrogenic PGF2α pathway in mare endometrium. Submitted to *Journal of Immunology Research* (manuscript 4806546).

5) To evaluate the involvement of PGE2 pathway in collagen deposition on mare endometrium, challenged with NETs proteases and if that effect could change with endometrial inflammatory/fibrotic lesions or with estrous cycle phase.
The developed work will be submitted for publication: Rebordão, M.R., Amaral, A., Lukasik, K., Szóstek-Mioduchowska, A., Pinto-Bravo, P., Galvão, A., Skarzynski, D.J. & Ferreira-Dias, G. (2017). Impairment of anti-fibrotic PGE_2 pathway might influence neutrophil extracellular traps-induced fibrosis in mare endometrium. Submitted to *Journal of Immunology Research* (manuscript 2053692).

Chapter II- *State of the Art*

1. Endocrine physiological events in mare endometrium

The equine estrous cycle is defined as the repetitive sequence of events that prepare the mare for conception, lasting on average 21 days. The mare estrous cycle can be divided in four stages according to the changes in steroid hormones concentration and endometrial structural and functional events: follicular phase (estrous/ovulatory phase; day 16-17 to 21, the latter usually being called day 0), early luteal phase (day 1 to 4), mid luteal phase (around day 8) and late luteal phase (day 12 to 15) (Aurich, 2011).

Estrus is the period during which the mare is sexually receptive to the stallion, the ovulatory (dominant) follicle(s) develop and ovulation occurs. During follicular phase, estrogens (E2) produced in the ovarian follicles are the main steroids that dictate the different physiological events that occur in the female body, specifically in the uterus. Under the influence of E2 during estrus, the uterine wall thickens, muscular tone increases and vascularity becomes greater. The cervix is relaxed and open (Aurich, 2011). Histologic observation of the mare endometrium in this stage depicts that endometrial glands proliferate and become active and the lamina propria is highly edematous. This E2-induced edema leads to reduced gland density and a loosely woven appearance of the stroma (Kenney, 1978). Neutrophils (PMN) can be seen to marginate venules and capillaries, but they do not migrate through the stroma. Therefore, the presence of neutrophils (PMN) in the stroma is indicative of inflammation (Kenney, 1978). At a molecular level, E2 stimulates or upregulates its own receptors (ESR), as well as the progesterone (P4) receptors (PGR) in mare's endometria (Hartt *et al.,* 2005). Estrogen receptors (ESR) consists of two predominant isoforms of nuclear receptors, ESR1 and ESR2. ESR1 appears to be the dominant mediator of uterine reproductive functions. Some studies in other species than the mare indicate that ESR2 may function to attenuate the transcriptional activity of ESR1 in the uterus (Large & DeMayo, 2012). In the mare, during estrus, high endometrial levels of ESR1, ESR2 and PGR mRNA and proteins are detected in luminal (LE) and glandular epithelia (GE), and stromal cells (Watson *et al.*, 1992; Hartt *et al.,* 2005; Honnens *et al.*, 2011; Gebhardt *et al.*, 2012; Silva *et al.*, 2014). Moreover, relative mRNA expression of *ESR1* and *ESR2* seems to be positively correlated in mare endometrium (Honnens *et al.*, 2011). In mare endometrium OXT receptors (OXTR) are low at estrus (Sharp *et al.,* 1997; Ruijter-Villani *et al.,* 2014).

Diestrus is the period during which the mare is not receptive to the stallion and the genital tract prepares for the reception and nurturing of the conceptus. During diestrus, the ruptured ovulatory follicle develops into a *corpus luteum* (CL) that secretes P4 and, in the non-pregnant mare, it regresses 14-15 days after ovulation. During the luteal phase, P4 becomes

the main steroid hormone influencing uterine environment. During this stage, the influence of P4 leads to a decrease in uterine wall thickness, myometrial tone and endometrial gland activity. The cervix becomes firmer and is tightly closed (Aurich, 2011). Histologically, during diestrus, gland density increases due to decreased stromal edema and the increased tortuosity of glands (Kenney, 1978). Organelles increase in number and size in stromal and glandular epithelial cells, indicating active protein synthesis during this stage of the cycle (Ferreira-Dias *et al.* 1999). At a molecular level, when circulating P4 levels are high, endometrial expression of ESR and PGR are inhibited. Concentration of E2 and P4 receptor proteins decreases by mid to late diestrus in mare endometrium (Watson *et al.*, 1992). Likewise, abundance of *ESR1* and *PGR* mRNA decreases from days 0 to 11 of estrous cycle and increases by late diestrus (McDowell *et al.*, 1999; Honnes *et al.*, 2011; Gebhardt *et al.*, 2012; Silva *et al.*, 2014). In pregnancy, gene transcription for both types of receptors decreases with increasing days of gestation and sustained P4 concentration (Watson *et al.*, 1992; McDowell *et al.*, 1999). In the presence of P4, a negative-feedback loop down-regulates the expression of PGR. On day 11 and 14 of mare estrous cycle, ESR and PGR expression decreased in stroma and in deeper glandular epithelia and they were not detected in luminal epithelium (Hartt *et al.*, 2005). On days 14 to 16 of mare estrous cycle luteolysis occurs (Ginther *et al.*, 2011). Then, oxytocin exerts a variety of physiological processes including luteal function regulation. Oxytocin is stored in vesicles in the neurohypophysis and released into the peripheral blood circulation in high frequency bursts in late diestrus (Vanderwall *et al.*, 1998). This hormone is also synthesized and secreted by mare endometrial cells into the uterine lumen where it acts in an autocrine/paracrine fashion to control uterine contractility, and luteolysis (Watson *et al.*, 2000). In late diestrus, *OXT* gene expression increases in the equine endometrium (Behrendt-Adam *et al*., 1999). In the ewe, during late diestrus (day 12–13 of estrous cycle), E2 interacts with P4 primed uterus and increased expression of ESR1, stimulates transcription of OXTR gene and increases the concentration of this receptors in the endometrium (Fleming *et al*., 2006). Although follicles and estradiol are apparently not required for luteolysis in mares (Ginther *et al.*, 2005), in all other mammals, E2 is considered a key regulator of *OXTR* gene expression (Kimura *et al.*, 2003). Besides, in the mare estradiol-17β begins to increase in the circulation approximately 2 days before the beginning of luteolysis and the start of growth rates deviation between the future dominant *versus* subordinate follicles of the ovulatory follicular wave (Ginther *et al.*, 2005). Oxytocin receptor activation stimulates the mitogen activated protein kinase (MAPK), which regulates prostaglandin-endoperoxide synthase *(PTGS)* enzymes gene expression (Soloff *et al.*, 2000). These enzymes convert arachidonic acid to prostaglandin H2 which can be

converted to $PGF_2\alpha$ by aldo-keto reductase family 1 member C3 (AKR1C3) in the uterus. At Day 15 in equine cycling endometrium raises in both mRNA and protein levels of PTGS2 in luminal epithelium were detected (Boerboom *et al.*, 2004; Atli *et al.*, 2010). In late luteal phase (days 14-15), cyclic mare endometrial OXTR concentration increases and mediates prostaglandin release (Ruijter-Villani *et al.,* 2014). Oxytocin binding to OXTR stimulates the concentration and amplitude of $PGF_2\alpha$ peaks from the endometrium, which are necessary for luteolysis (Sharp *et al.*, 1997; Starbuck *et al.,* 1998). In response to luteolytic pulses of $PGF_2\alpha$ over a 23-h period, the CL undergoes functional and structural regression and P4 progressively decreases to < 1ng/mL (Ginther *et al.*, 2011). Between days 17 to 20 of cyclic mares, *ESR* and *PGR* transcripts and proteins increased and were present in luminal epithelium, glandular epithelium and in stroma cells of the endometrium (Hartt *et al.*, 2005). Nevertheless, during early pregnancy, ESR and PGR were not detected in the luminal epithelium, and were lower in the stroma and deeper glandular epithelium (Hartt *et al.*, 2005). So, the development of the endometrial luteolytic mechanism and uterine release of luteolytic pulses of $PGF_2\alpha$ occur in the presence of high circulating levels of P4. As suggested for the ewe (Fleming *et al.*, 2006), in the mare, the lack of response to luteolytic mechanisms during luteostasis periods (days 7-14 of estrous cycle and in early pregnancy) may be due to the absence of ESR and PGR in luminal epithelium.

The deep knowledge of cellular and molecular mechanisms, including the cross-talk between different cells, hormones and receptors that control the estrous cycle, allows its manipulation and is the basis of several reproductive techniques namely of several methods of estrus suppression. An example is given by the dual and antagonist role that oxytocin may have in the mare reproduction. Endogenous or exogenous oxytocin has a pro-luteolytic effect between days 10 and 15 after ovulation, while its acts as an anti-luteolytic factor during mid-luteal phase (Rebordão *et al.*, 2016; Vanderwall *et al.*, 2016).

2. Estrus behavior suppression

Veterinary practitioners are often requested by horse mare owners to suppress estrus behavior during sports activities and other events, since undesirable and/or inconstant behavior in mares in estrus can interfere with and decrease their performance. Although several methods can be used to suppress estrus behavior, they should not affect athletic performance of the mare, and they should have no effect on the mare´s reproductive efficiency during and subsequently to their athletic careers (Nath, 2014). Artificial extension of the luteal phase by

luteolysis inhibition and subsequent suppression of heat signs in the mare can be achieved by non-pharmacological or pharmacological methods.

Manual reduction of the conceptus and insertion of intrauterine devices are non-pharmacological methods used to suppress estrus behavior in mares. Embryo manual crushing on day 16–22 post-ovulation, is a method that allows the maintenance of the CL, despite embryo loss, and suppresses estrus behavior for at least 60 days (Lefranc & Allen, 2004). Considerable investment in first establishing a pregnancy and ethical issues are constraints that limit its use (Vanderwall, 2013; Nath, 2014).

Insertion of intrauterine devices (IUD) like glass balls or water-filled plastic balls is the most common method used for preventing estrus signs in the mare that does not require administration of exogenous hormones, and allows continued secretion of endogenous P4 to keep mares out of heat (Nie *et al*., 2003; Rivera del Alamo *et al*., 2008). Prolonged CL function was achieved in 39% of the mares that retained the glass balls inside the uterus and the duration of estrus suppression was approximately 90 days (Nie *et al.*, 2003). Likewise, after intrauterine placement of a water-filled plastic ball, 75% of the mares had an extended luteal function with an average duration of 57 days (Rivera del Alamo *et al*., 2008). The efficacy of IUD seems to be dependent on the mares´ age. Intrauterine device-treated mares with extended luteal function were younger than those without CL prolongation (Katila, 2015). A moderate degree of angiopathy was detected in some of the mares that did not respond to IUD with CL prolongation, suggesting that adequate perfusion and drainage of the endometrium may be important for the IUDs to function effectively (Katila, 2015). It has been suggested that IUD can mimic an embryo by simulating maternal recognition of pregnancy (that occurs when a mobile embryo is present in the uterus prior to day 14 post-ovulation), suppressing $PGF_{2\alpha}$ and inducing CL maintenance (Rivera del Alamo *et al*., 2008). There was no evidence that IUD induced an inflammatory response in the uterus (Rivera del Alamo *et al*., 2008). In addition, in the mares that develop prolonged CL function, the IUD prevented up-regulation of endometrial gene expression of PTGS2, which is the key regulatory enzyme in PGF2α synthesis/secretion (Rivera del Alamo *et al*., 2010). While the placement of these IUD does not seem to have an adverse effect on fertility (Nie *et al*., 2003; Rivera del Alamo *et al*., 2008), severe complications can result if they are retained for a prolonged time, like fibrosis of cervical canal due to cervical adhesions of fragmented glass ball and pyometra (Klabnik-Bradford *et al*., 2013, Turner *et al*., 2015).

Intrauterine infusion of plant oils has also been proposed as a possible reversible method for prolonging the luteal phase in mares, but further studies are needed to confirm its efficacy. In a previous study, intrauterine infusion of plant oils on day 10 post-ovulation prolonged luteal

phase in 92% of the treated mares (Wilsher & Allen, 2011). It was suggested that the fatty acid milieus in plant oils might modulate or attenuate the synthesis and/or secretion of PGF2α at the expected time of luteolysis, resulting in prolonged function of the CL (Wilsher & Allen, 2011). Nevertheless, a recent report failed to replicate those results: intrauterine fractionated coconut oil administration on day 10 of diestrus lowered P4 levels and shortened rather than prolonged the luteal phase of the mares (Diel de Amorim *et al*., 2016).

Pharmacological prevention of luteolysis in the mare by maintaining the secretion of P4 from the CL may be achieved by the use of exogenous administration of human chorionic gonadotropin (hCG), P4, synthetic progestagens or oxytocin. Seventy five percent of the mares treated with hCG ovulated within 72 hours after treatment with hCG, which resulted in prolonged luteal phase that lasted for 58 to 82 days after treatment (Hedberg *et al*., 2006). However, the use of hCG to induce a late diestrus ovulation does not appear to be a reliable method of blocking estrus behavior in mares (Vanderwall, 2013). Exogenous hCG has to be administrated to mares in diestrus when a follicle greater than 30 mm is detected in the ovaries. In addition to the need to continually monitor mares to evaluate their suitability for treatment, some mares may not develop a diestrus follicle large enough to be eligible for treatment (Vanderwall, 2013).

For the past decades, P4 or progestogen have been used to control estrus behavior. Daily administration of altrenogest, a synthetic progestin, has been the most widely used method and it is considered to be the 'gold standard' for suppressing estrus behavior in mares (Nath, 2014; Vanderwall *et al*., 2016). Besides being expensive, the need for oral administration of altrenogest every 24h, encompasses safety risks for personnel during handling, and there is a risk of development of putative uterine infection in the mare due to maintenance of a closed cervix. All of these are limitations for long-term progestin therapy (Vanderwall, 2013; Nath, 2014; Vanderwall *et al*., 2016). Additionally, concerns regarding the use of exogenous steroid hormones in performance horses and stricter doping regulations, has led to the study of alternative methods.

Administration of exogenous OXT on days 7 to 14 after ovulation is an effective method of disrupting luteolysis and prolonging CL function that can be used as a means of suppressing estrous behavior in mares (Stout *et al*., 1999; Vanderwall *et al*., 2007; Gee *et al*., 2012; Keith *et al*., 2013; Vanderwall, 2012). Intramuscular (im) administration of 60 units of oxytocin every 12 h prolonged luteal function in 70% to 100 % of the treated mares (Vanderwall *et al*., 2007; 2012; Keith *et al*., 2013). It has been proposed that once-daily im administration of 60 IU of OXT during this phase of the cycle is as effective as the twice-daily protocol first described, inducing prolonged CL in 60% to 70% of treated mares (Vanderwall *et al*., 2016).

To preclude the need to determine the exact day of ovulation before initiating treatment, administration of OXT im can be initiated randomly at any point in the estrous cycle with no loss in efficacy (i.e., over 70% response) extending the duration of OXT treatment to 29 days (Vanderwall *et al*., 2016). Although OXT treatment effectively prolonged CL function, a weak estrus behavior was occasionally observed in some mares during the period of prolonged CL function Vanderwall, 2013). It has been proposed that OXT treatment suppresses PGF2 α secretion by preventing upregulation of endometrial gene expression of PTGS2 (Keith *et al*., 2013). An advantage of using OXT treatment to prolong CL function is that it can be readily reversed by the administration of a PGF2α luteolytic dose to initiate cyclical reproductive activity. The main disadvantage of the OXT protocol is the daily administration of an intra-muscular injection, leading to potential difficulties with its use in mares with a needle aversion (Vanderwall *et al*., 2016). No side effects were reported regarding body temperature, heart rate or respiratory rate treatment (Vanderwall, 2013). There was no evidence of abdominal cramping and/or transient signs of colic before or after the treatment (Vanderwall, 2013). However, mild sweating, urticaria around the injection site, and mild diarrhea have been referred in some mares treated with 60 units of OXT (Vanderwall, 2013).

3. Neutrophils

Neutrophils (PMN) are the most abundant inflammatory cells produced in the bone marrow from myeloid precursors (Kazzaz *et al*., 2016; Nakazawa *et al*., 2017). They are involved in host defense at the inflamed tissues (reviewed by Kazzaz *et al*., 2016; reviewed by Nakazawa *et al*., 2017). In healthy humans, PMN are the major leukocytes (40–80%) in peripheral blood and the biggest PMN pool in the organs is in the lungs, where they act as sentinels against infection (Lacy, 2016). The ability of PMN to rapidly localize the sites of tissue inflammation and subsequently capture and destroy the invading pathogens is a hallmark of the highly conserved innate immune response (Zinkl & Kabbur 1997; Brinkmann, 2011; Oliveira *et al*., 2016). While PMN circulate in the blood stream, they respond to inflammatory stimuli by migrating to the sites of inflammation in various tissues (Kazzaz *et al*., 2016). These extremely well-coordinated events rely on the interaction of adhesion molecules on the PMN surface with their respective ligands existing on the vascular endothelium (Kazzaz *et al*., 2016). In order to cross the endothelial barrier, PMN develop cytoplasmic membrane extensions that help these cells to overcome the high shear stress of blood flow (Sundd *et al*., 2012). Then, PMN β2 integrins (LFA-1 and Mac-1) firmly adhere to endothelial cell

intracellular adhesion molecule 1 (ICAM-1) and ICAM-2 and cross the endothelium barrier (Sundd *et al*., 2012; reviewed by Kazzaz *et al*., 2016).

In the context of inflammatory cues, PMN express over 30 different types of receptors that can detect pro-inflammatory factors that mediate PMN migration to the inflammation site and their function (Oliveira *et al*., 2016). Among those pro-inflammatory receptors are included the G protein-coupled receptors (GPCRs), Fc receptors, adhesion receptors, cytokine receptors, as well as pattern recognition receptors (PRRs) (Futosi *et al*., 2013). In PMN, the signaling vias downstream of G protein-coupled receptors that can sense pro-inflammatory cues, are involved in PMN gene transcription modulation, phagocytosis, apoptosis, degranulation and reactive oxygen species (ROS) production (Sun & Ye, 2012). The recruitment of PMN to the site of tissue damage is a complex and well-regulated process that occurs in three major phases, as reviewed by Oliveira and co-authors (2016). Understanding this biological process becomes even more challenging, when based on some evidence that PMN migration differs in response to signals produced either by the host or by the pathogen (Deng *et al.*, 2012; Yan *et al*., 2014). In the early phase, as soon as tissue injury happens, short-lived signs sent from the damaged cell trigger the release of damage-associated molecular patterns (DAMPs) that activate hydrogen peroxide (H_2O_2) production and stimulate the chemotaxis of PMN present in the neighboring cells previously recruited through the SRC family kinase LYN (Oliveira *et al*., 2016). After initiating the migration of resident PMN, DAMPs further stimulate the production of CXC motif chemokine ligand 8 (CXCL8) family chemokines, as well as other pro-inflammatory cytokines, interleukins (IL-1β) and lipid mediator leukotrienes (LTB4) from the surrounding tissues to attract other early PMN (Oliveira *et al.*, 2016). These previously recruited PMN present in the tissue, are continuously activated by LTB4 or by IL-1β that increases CXCL8 family chemokines. In addition, necrotic PMN, as occurs in damaged tissue in the initial phase of PMN recruitment, also cause further DAMPs release after cell damage that will modulate transcriptional activation of tissue cells and CXCL8 release (Oliveira *et al*., 2016). Both CXCL8 and LTB4 will further attract PMN from blood circulation and amplify this recruitment process. When an infection is present, additional levels of signaling are needed to extend and intensify PMN recruitment. In contrast to the initial stage of aseptic tissue injury, hydrogen peroxide release is not a required signal for PMN to detect and localize the infection by *Pseudomonas aeruginosa,* Gram-negative bacteria (Deng *et al*., 2012). When an infection is present, the release of pathogen-associated molecular patterns (PAMPs) and the involvement of other immune cell-like tissue residents and recruited inflammatory macrophages (LY6C), T cells and dendritic cells are mandatory (Oliveira *et al*., 2016). As a result, pro-inflammatory cytokines such as

IL-1β and TNFα stimulate CXCL8 family chemokines and leukotrienes release, prolonging and amplifying PMN recruitment (Oliveira *et al*., 2016). As a major pro-inflammatory cytokine, TNF-α triggers a cascade of events, including the production of other cytokines and chemokines by mast cells and resident tissue macrophages, which attract PMN to the inflammatory site. This cytokine also induces a stop signal that results in PMN firm adhesion to blood vessels´ endothelium (Locksley *et al*., 2001; Wiemer *et al*., 2010; Souza *et al*., 2011). This enables PMN to cross this cell barrier and reach the site of infection, being PMN polarization and chemotaxis inhibited (Wiemer *et al*., 2010), while some of their other functions like oxidative burst (Nathan, 1987), phagocytosis and degranulation are activated (van der Poll *et al*., 1992). Since the inflammatory process should be sustained until the infection no longer exists, this time frame does influence the duration of tissue damage (Oliveira *et al*., 2016). Matrix metalloproteinases (MMPs) expressed in activated PMN, may also enhance PMN chemotaxis by cleaving CXCL8 family chemokines or by acting on collagen itself to release collagen-derived chemotactic peptides (Tester *et al*., 2007; Afonso *et al*., 2013).

A purposeful response to acute injury is resolving inflammation *in situ* through PMN clearance to avoid tissue damage (Oliveira *et al*., 2016). Nevertheless, PMN appear to have a dual effect: while their aim is to fight inflammation or infection, they themselves trigger an inflammatory response by undergoing receptor mediated respiratory burst and degranulation (Lacy, 2006). Even though PMN removal can be achieved through apoptosis, necrosis or macrophage phagocytosis, some PMN do not undergo apoptosis at inflamed tissues. Instead, they leave inflamed and damaged tissues in a process called “neutrophil reverse migration”. This consists on a retrograde chemotaxis back toward the main circulation, as it was first visualized *in vivo* in zebrafish larvae (Mathias *et al*., 2006). It is worth noting that many aspects of “neutrophil reverse migration” are still very challenging and yet to be understood, such as the fate of those PMN and its role in disease.

4. Neutrophil Extracellular Traps

Once in the inflamed tissue, PMN purpose is to kill the invading pathogen. They do so through several mechanisms, such as the extracellular release of lytic enzymes and phagocytosis (Neeli *et al.*, 2009; Souza *et al*., 2011). As a new paradigm of innate immunity, PMN were found to have the ability to extrude their deoxyribonucleic acid (DNA) in response to infectious stimuli to form web-like structures called neutrophil extracellular traps (NETs) (Brinkmann *et al*., 2004). This finding has proved that other mechanisms of the innate

immune system, rather than PMN classical functions of extracellular release of lytic enzymes and phagocytosis, should be involved in NETs formation (Neeli *et al*., 2009; Brinkmann *et al*., 2004; Brinkmann, 2011; Nakazawa *et al*., 2017). Although NETs are widely used to combat infection, their antimicrobial mechanism remains an enigma (Halverson *et al*., 2015). Besides the antimicrobial activity attributed to the nucleic and cytoplasmic proteins bound to the DNA backbone, it appears that DNA itself contributes for the antibacterial nature of NETs (Halverson *et al*., 2015). However, it might also act as a signal perceived by microbes to elicit host-resistance strategies (Halverson *et al*., 2015).

The word NETosis was initially proposed as the release of NETs by PMN with their death. Nevertheless, PMN stimulated by pathogens are also able to induce a rapid and vital form of NETosis, in which the PMN keep their phagocytic, chemotaxis, and bacteria killing functions after producing NETs (Jorch & Kubes, 2017). The stimulus seems to play an important part in determining which kind of NETosis is induced (Jorch & Kubes, 2017). In induced "suicidal NETosis", which occurs after hours of stimulation, like with phorbol-myristate acetate (PMA), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is activated, ROS are produced and protein-arginine deiminase 4 (PAD4; an enzyme that converts arginine to citrulline on histones) is activated, which results in chromatin decondensation. Further unfolding of chromatin is promoted by translocation of PMN elastase (ELA) and myeloperoxidase (MPO) into the nucleus with disruption of the nuclear membrane. Chromatin is then released into the cytosol, where it becomes decorated with granular and cytosolic proteins. After disruption of the plasma membrane NETs are released, forming a netlike structure outside the cell, and the PMN die (Jorch & Kubes, 2017). "Vital NETosis" occurs very quickly after stimulation of PMN with bacteria or bacterial products. This activation can be done (i) through both complement receptors and TLR2 ligands (with *Staphylococcus aureus)*, (ii) directly via TLR4 (with *Escherichia coli*) or (iii) indirectly via TLR4-activated platelets. PAD4 is activated, perhaps without any need for oxidants, and induces chromatin decondensation. Elastase is translocated into the nucleus to promote further unfolding of chromatin and nuclear-membrane disruption. The chromatin, in direct contact with PMN proteins, is expelled via vesicles, and the PMN stay alive for further functions. How mitochondrial ROS production is involved in one or the other form of NETosis has not yet been completely elucidated (reviewed by Jorch & Kubes, 2017). Nevertheless, the term "Vital NETosis" is still under debate because it is not clear, if PMN actually remain alive thereafter (Zawrotniak *et al*., 2017).

As a result of NETosis, NETs proteins components, like histones and enzymes as elastase (ELA), proteinase 3, cathepsin G (CAT) and myeloperoxidase (MPO), may be involved in

microorganism killing (Weinrauch *et al*., 2002; Wartha *et al.*, 2007; Brinkmann, 2011; Windt *et al*., 2012; Marin-Esteban *et al*., 2012). In addition, besides having potent antimicrobial functions, the proteins forming NETs might also have immune-modulating capacities (Lögters *et al.,* 2009). Among the pathogens that induce NETs release, bacteria (Fuchs *et al*., 2007, Pilsczek *et al*., 2010; Rebordão *et al*., 2014; Rebordão *et al*., 2017b), Floyd *et al*., 2016), protozoa (Guimarães-Costa *et al*., 2009, Wardini *et al*., 2010; Abdallah *et al*., 2012), or yeast, have been reported (Urban *et al*., 2006, 2009). Interestingly, in some bacteria, like the *Pseudomonas aeruginosa*, the flagellar motility is required for the induction of NETs, rather than the binding of the flagellum to host cells sensing receptors (Floyd *et al*., 2016). Thus, at localized inflammation/infection sites, NETs are abundant. They have been found in spontaneous appendicitis in humans, endometritis in the mare, pyometra in the queen and bitch, peritoneal fluid of endometriosis human patients, mastitis in sheep, or skin infection in mice (Brinkmann *et al*., 2004; Berkes *et al*., 2014; Rebordão *et al*., 2014; 2017b; Pisanu *et al*., 2015; Halverson *et al*., 2015). Besides PMN response to infectious agents, also many physiologic inducers of NETs formation, like sterile stimulation with chemicals or cytokines have been reported (reviewed by Brinkmann & Zychlinsky, 2012; Kazzaz *et al*., 2016).

Apparently, NETs may act as a physical barrier to prevent further spread of microorganisms that get trapped at the infection site, limiting bacterial dissemination keeping an abscess from becoming bacteremic or confining tissue infection to local sites (Beiter *et al*., 2006; O'Brien *et al.*, 2017). Although PMN are not capable of directly catching circulating pathogens, by producing NETs, they can increase the catching capacity of the liver (Kolaczkowska *et al.,* 2015). It has been suggested that a major role for NETs is to trap rather than kill microbes since the majority of bacteria bound to NETs were alive when liberated by DNase treatment (Menegazzi *et al.,* 2012). Whenever NETs release is not physiologically controlled, it can be a major trigger of tissue injury or can exacerbate the disease as in some cancers, metabolic disorders, autoimmune diseases, and thrombotic diseases (Corsiero *et al*., 2016; Nakazawa *et al*., 2017). The formation of NETs may be also mediated by immunoglobulin A immune complexes, as occurs in humans with joint damage due to rheumatoid arthritis, thereby increasing the severity of the disease (Aleyd *et al*., 2016). While deamination of histones present in NETs is a physiological mechanism, this process may be intensified under inflammatory conditions (Corsiero *et al*., 2016). In some individuals carrying a genetic predisposition to develop auto-immune diseases, such as rheumatoid arthritis, NETs themselves modulate the production of self-antigens, and contribute to the expansion and differentiation of antibody producing B-cells (Corsiero *et al*., 2016). Thus, antibody-antigen

complexes are among those NETs inducers that maintain an inflammatory milieu propitious for further NETs stimulation (Garcia-Romo *et al*., 2011; Corsiero *et al*., 2016).

While NETs can disarm both Gram-positive and Gram-negative pathogens (Brinkman *et al.,* 2004), PMN appear to discriminate between different Gram-negative bacteria lipopolysaccharide (LPS), depending on species-specific bacteria and also on serotype-specific bacteria (Pieterse *et al*., 2016). Human saliva mucin is also capable of inducing NETs. This mucin-induced NETs have a higher capacity to attach and destroy bacteria and they are more DNase-resistant than NETs induced by bacteria *Staphylococcus aureus* and *Streptococcus pyogenes* or by PMA (Mohanty *et al*., 2015). Saliva-induced NETosis is dependent on the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway but not on NADPH oxidase or elastase (Mohanty *et al*., 2015). Depending on the affected organ, NETs persistence and amount (acute *versus* chronic process), NETs can either fight disease or cause disease themselves (Brinkmann & Zychlinsky, 2012). However, despite all the research carried out on NETs, their dual and somewhat antagonist functions-antimicrobial and pro-inflammatory actions - remain an enigma.

5. Neutrophil Extracellular Traps (NETs) and NETs components in fibrosis formation

The connection between inflammation and fibrosis is well recognized and non-resolving inflammation after chronic injury is seen as a major driving force in the development of fibrotic disease (Liu 2011; Zeisberg & Kalluri, 2013; Wynn & Vannella, 2016). Although uncontrolled NETs release has been linked to the development of several disease conditions (reviewed by Jorch & Kubes, 2017; Nakazawa *et al*., 2017) the role of NETs in fibrosis has recently been established.

In an *in vitro* study, NETs released by PMN treated with different fibrosis-related agents, triggered the activation and differentiation of lung fibroblasts into myofibroblast phenotype. This was significantly decreased after degradation of NETs with different agents (Chrysanthopoulo *et al*., 2014). Also, IL-17 expression in NETs promoted the fibrotic activity of myofibroblasts but not their differentiation, suggesting that priming by NETs components like DNA, histones and MPO is essential for IL-17-driven fibrosis. The role of PMNs in fibrogenesis in those *in vitro* findings was further supported by the identification of NETs near α-smooth muscle actin (α-SMA)-positive fibrocytes in biopsies from human patients with fibrotic interstitial lung disease or from skin scar tissue (Chrysanthopoulou *et al*., 2014). It was proposed that PMN infiltration in tissues affected by chronic inflammation, caused by either pathogens or environmental agents, may perpetuate tissue injury through NETs release.

NETs components, including histones, antimicrobial peptides, and cytokines, in conjunction with a possible defect in NETs clearance by either DNase or macrophages may impact fibroblast activation, contributing to disease progression towards fibrosis (Chrysanthopoulou *et al*., 2014). In a murine model of atherosclerosis, cholesterol crystals trigger PMN to release NETs, which prime local macrophages to transcribe immature IL-1β. Cholesterol crystals serve a second role as a danger signal that activates inflammasomes, which process immature IL-1β secretion. NETs prime macrophages for cytokine release and activate T-helper type 17 (Th-17) cells that amplify immune cell recruitment in atherosclerotic plaques (Warnatsch *et al.,* 2015).

Persistence of other NETs components in chronic inflamed tissues, like NETs proteases may also be involved in fibrogenesis. NETs are a major contributor to chronic inflammation and lung tissue damage in chronic obstructive pulmonary disease human patients (Grabcanovic-Musija *et al*., 2015). Uncontrolled protease release by PMN contributes to the destruction of lung tissue in cystic fibrosis (Dubois *et al*., 2012). The dominant proteolytic activity in NETs was attributed to ELA, but when ELA was immune-depleted, the remaining activity was attributed to CAT (O'Donoghue *et al.,* 2013). The NETs released in the liver vasculature remain anchored to the vascular wall. In this case, liver injury was attributed to PMN-ELA and a large portion (at least 80%) of that damage was due to NETs production (Kolaczkowska *et al.,* 2015). The effectiveness of DNase treatment seems to be limited in terms of removal of the most dangerous NETs components, since the removal of DNA with DNase failed to remove all histones or proteases from the vascular wall (Kolaczkowska *et al.,* 2015).

Neutrophil-derived ELA has been shown to play a pivotal role in the pathogenesis of pulmonary fibrosis (Takemasa *et al*., 2012). In the early-stage of a pulmonary fibrosis model, the ELA level was increased in the lungs and a PMN-ELA inhibitor (Sivelestat) inhibited the fibrotic changes, the numbers of total cells (including macrophages, PMN and lymphocytes) and the levels of the active form of transforming growth factor-β1 (TGF-β1) and phospho-Smad2 (Takemasa *et al*., 2012).

Cathepsin G (CAT) was also shown to be associated with the formation of atheromas in human carotid arteries (Legedz *et al.,* 2004). In stenotic valves, CAT was present in mast cells and showed increased expression which correlated positively with the expression levels of TGF-β1 and type I and III collagens (Legedz *et al.,* 2004). Cathepsin G is capable of generating angiotensin II, a powerful pro-fibrotic and pro-inflammatory mediator in the pathogenesis of aortic stenosis, characterized by extensive remodeling of the valves, including infiltration of inflammatory cells, extracellular matrix degradation, and fibrosis (reviewed by Helske *et al*., 2006)

Neutrophil ELA and CAT are proteolytic activators of MMP-9 (Vandooren *et al*., 2013). Additionally, MMPs (particularly MMP-2 and MMP-9) enhance the release of TGF-β1. TGF-β1 stimulates tissue inhibitor of metalloproteinase (TIMP), resulting in inhibition of extracellular matrix (ECM) degradation, which further induces ECM accumulation, vascular remodeling and vascular fibrosis (Harvey *et al.,* 2016).

6. Cellular and molecular mechanisms of tissue fibrosis

Fibrogenesis is the deposition of pathological extracellular matrix (ECM) by cells. It is widely believed to be a wound-healing response to tissue injury that does not resolve (Zeisberg & Kalluri, 2013; Duffield, 2014; Wynn & Vannella, 2016). Excessive deposition of ECM leads to the destruction of organ architecture and impairment of organ function. Chronic loss of organ function in most organs, including bone marrow, heart, intestine, kidney, liver, lung and skin is associated with fibrosis (Zeisberg & Kalluri, 2013).

Fibrosis is a very complex pathology since different etiologies and pathogenesis are involved in organ fibrosis. It is triggered by activation of different type of cells into myofibroblasts and different molecular and immunological mechanisms that initiate, maintain and terminate the differentiation of quiescent fibroblasts into actively proliferating ECM-producing myofibroblasts (Wynn & Ramalingam, 2012; Duffield, 2014; Wynn & Vannella, 2016).

The main source of collagen in fibrosis are "activated fibroblasts" (reflecting their increased biosynthetic and proliferative activities). Phenotypically, activated fibroblasts are characterized by a pronounced rough endoplasmic reticulum, stress fibers, and a large nucleolus. Since the observation that the majority of collagen-producing fibroblasts are labeled with antibodies to the filament α-smooth muscle actin (α-SMA) (Kalluri & Zeisberg, 2006), the terms "activated fibroblasts" and "myofibroblasts" are used as synonymous. They both refer to the fibroblasts that mediate fibrosis (Zeisberg & Kalluri, 2013). However, α-SMA expression is an imperfect marker for such an important cell type (Liu, 2011; Duffield, 2014), since is not exclusive to myofibroblasts, as it is also present in vascular smooth muscle cells (Liu, 2011). Nevertheless, the majority of cells that produce collagen type I are shown to also express α-SMA and abundance of α-SMA is closely correlated with the severity of fibrosis in several organs (Liu, 2011, Duffield, 2014). Other markers have been proposed to characterize myofibroblasts, including fibroblast specific protein–1 (FSP-1), heat shock protein–47, and vimentin, although none of these markers is specific (Liu, 2011; Duffield, 2014).

While there is a consensus that (activated) fibroblasts are the principal source of collagen and prominent mediators of fibrogenesis, it is not yet clear where these activated fibroblasts

originate from (Zeisberg & Kalluri, 2013). Contribution to fibroblast accumulation and fibrogenesis may differ substantially among organs, and disease models (Zeisberg & Kalluri, 2013). Although resident fibroblasts are important cells by their own activation and proliferation, other cells also contribute to fibroblast accumulation. Among them are bone marrow-derived fibrocytes, vascular smooth muscle cells, pericytes and transdifferentiation of epithelial cells into epithelial-mesenchymal transition (EMT) and of endothelial cells into endothelial-mesenchymal transition (Liu, 2011; Ueha *et al.*, 2012; Zeisberg & Kalluri, 2013). The relative contribution, and even the very existence, of each particular myofibroblast-generating pathway to fibrosis is a matter of intense discussion (Liu, 2011). This is due to the difficulty in identifying and tracking fibroblasts owing to the lack of specific markers for this cell type (Liu, 2011). In addition, fibroblasts exhibit enormous phenotypic heterogeneity, probably reflecting their diverse origins, activation status, localization and stage of fibrogenesis (Liu, 2011).

The classic view on the relationship between inflammation and fibrosis is that they are mediated in a paracrine fashion, whereby inflammatory cells secrete pro-fibrotic cytokines that act on resident fibroblasts and tubular cells to promote fibrogenesis (Liu, 2011). The cross-talk of the injured epithelium with fibroblasts and inflammatory cells is of foremost relevance for triggering the fibrotic process (Zeisberg & Kalluri, 2013). In addition to the release of pro-fibrotic metabolites (i.e., reactive oxygen species - ROS), injured parenchymal cells govern fibrogenesis through secretion of chemokines and growth factors (Zeisberg & Kalluri, 2013). Nevertheless, intrinsic connection at the molecular level exists between inflammatory signals and fibrosis within the same cells (Liu, 2011). For instance, activation of nuclear factor kappa B (NFκB) that is dependent on tumor necrosis factor stabilizes Snail1 by blocking its ubiquitin-mediated degradation. As Snail1 is a key transcription factor that promotes EMT, fibroblast migration and renal fibrosis, this finding provides a molecular link between inflammatory signaling and fibrosis (Liu, 2011).

When tissues are injured during infection or following toxic or mechanical injury, an inflammatory response is induced to damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) released by dead and dying cells and invading organisms, respectively (Zhang *et al.*, 2010; Wynn & Vannella, 2016). These molecules induce a complex inflammatory response that is characterized by the recruitment, proliferation, and activation of a variety of hematopoietic and non-hematopoietic cells. Neutrophils, macrophages, innate lymphoid cells, natural killer (NK) cells, B cells, T cells, fibroblasts, epithelial cells, endothelial cells, and stem cells together make up the cellular response that conducts tissue repair (Wynn, 2008; Wynn & Vannella, 2016). When the wound

healing response is well organized and controlled, the inflammatory response quickly resolves and normal tissue architecture is restored. However, if the wound healing response is chronic or becomes dysregulated it can lead to the development of pathological fibrosis (Wynn & Vannella, 2016). Although inflammation is an integral part of the host defense mechanisms in response to injury, non-resolving inflammation after chronic injury is a major driving force in the development of fibrotic disease because it creates a vicious cycle of inflammation, tissue damage and fibrosis (Liu, 2011). Figure 1 depicts the complexity of fibrogenesis.

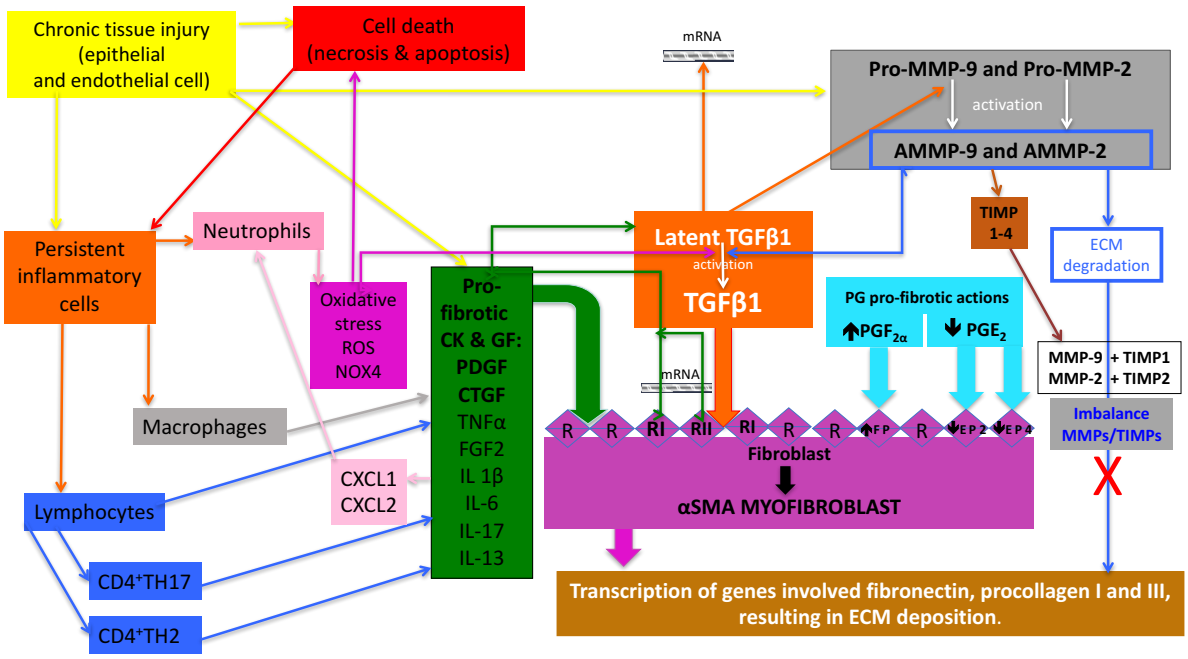

Figure 1- Schematic representation of some mediators and pathways involved in tissue fibrosis. AMMP- active matrix metalloproteinase; CTGF-connective tissue growth factor; CXCL- CXC motif chemokine; ECM- extracellular matrix; FGF- fibroblast growth factor; IL- interleukin; MMP- matrix metalloproteinase; NOX- NADPH oxidase; PDGF- platelet-derived growth factor; ROS- reactive oxygen species; TGF- transforming growth factor; TIMP- tissue inhibitor of metalloproteinase; TNF- tumor necrosis factor; SMA- smooth muscle actin.

6.1. Innate and adaptive immunity triggers fibrosis formation

Fibroblasts are triggered by components of the innate and adaptive immunity and upon activation they modulate immune cell behavior by conditioning cellular and cytokine microenvironment leading to chronic inflammation. Tissue damage, infections with bacteria, viruses, fungi, parasites, foreign body implants, autoimmune diseases, or tumors could progress to an adverse chronic inflammation, which subsequently leads to fibrotic disease (Van Linthout *et al.*, 2014).

The first-line defense of the innate immune system largely depends on a sophisticated array of pattern recognition receptors (PRR), which recognize conserved pathogen-associated molecular patterns (PAMPs) (Van Linthout *et al.*, 2014). Fibroblasts express a variety of pattern recognition receptors, including Toll-like receptors (TLRs), and activation of those receptors can directly activate fibroblasts and promote their differentiation into myofibroblasts (Van Linthout *et al.*, 2014).

Platelets also express a range of TLRs, contributing to their immune cell function in the state of infection and inflammation (Van Linthout *et al.*, 2014). Activated platelets by releasing several growth factors, like TGF-β1, platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) are also an important source of paracrine stimuli in fibroblast activation which stimulate the deposition of ECM (Van Linthout *et al.*, 2014; Elpek, 2014).

Neutrophils (PMN) act as a first-line defense and initiate an acute inflammatory response to engulf dead cells and tissue debris in order to facilitate tissue repair. However, excessive and persistent PMN infiltration or their delayed elimination exacerbate tissue injury by releasing inflammatory mediators and proteinases (Van Linthout *et al.*, 2014). Neutrophils release large amounts of ROS during the respiratory burst via the multicomponent enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Reactive oxygen species-released by PMN are a proven pro-fibrotic mediator in systemic sclerosis, pulmonary fibrosis, and hepatic fibrosis (Van Linthout *et al.*, 2014; Elpek, 2014). In fact, inhibition of NADPH oxidase attenuates cardiac fibrosis post-myocardial infarction (reviewed by Van Linthout *et al.*, 2014; Elpek, 2014). In addition, PMN secrete granules containing enzymes such as MMPs, elastase, and cathepsin capable of cleaving collagenous and non-collagenous connective tissue components, involved in tissue remodeling during the fibrotic process. In addition, neutrophil-derived elastase (NE) has been shown to play a pivotal role in the pathogenesis of pulmonary fibrosis (Takemasa *et al.*, 2012). Inhibition of NE suppressed both neutrophilic inflammation and TGF- β activation, which resulted in the suppression of pulmonary fibrosis (Takemasa *et al.*, 2012). Also, PMN by activating further cellular components of the innate immune system such as macrophages play an indirect pro-fibrotic effect (Van Linthout *et al.*, 2014).

Although many cell types are involved in tissue repair, because of their highly flexible programming, macrophages have been shown to exhibit critical regulatory activity at all stages of repair and fibrosis (Wynn & Vannella, 2016). Macrophages stimulated by TLR ligands and interferon (IFN)-γ undergo classical M1 activation, while those stimulated by IL-4 and IL-13 become M2 macrophages (Van Linthout *et al.*, 2014). M1 macrophages express IL-1, IL-12, IL-23, and induce T-helper type 1 (Th1) cell infiltration and activation (Ueha *et al.,* 2012). M2 macrophages express immunosuppressive molecules such as IL-10, which

suppress the induction of Th1 cells that produce the anti-fibrotic cytokine IFNγ (Ueha *et al.,* 2012). M2 macrophages are involved in wound healing, tissue remodeling, fibrosis, and inflammatory responses (Van Linthout *et al.*, 2014). Although pro-inflammatory and anti-inflammatory macrophages are two phenotypes involved in studies of wound repair and fibrosis it is not clear if they represent distinct subsets or whether it is the pro-fibrotic microenvironment that drives macrophage toward a M2 phenotype (Ueha *et al.,* 2012; Wynn & Vannella, 2016). Disturbances in macrophage function can lead to abnormal repair, with uncontrolled inflammatory mediator and growth factor production. Also, deficient generation of anti-inflammatory macrophages, or failed communication between macrophages and epithelial cells, endothelial cells, fibroblasts, and stem or tissue progenitor cells, may contribute to a state of persistent injury and lead to the development of pathological fibrosis (Wynn & Vannella, 2016).

Macrophages produce important pro-fibrotic mediators like TGF-β1 considered the most significant pro-fibrotic agent involved in the progression of chronic fibrotic diseases of several organs (Van Linthout *et al.*, 2014; Wynn & Vannella, 2016). Inhibition of alternatively activated macrophages (M2) has been shown to abrogate TGF-β-driven lung fibrosis (Van Linthout *et al.*, 2014). Activated macrophages also produce cytokines such as IL-1, IL-4, IL-10, IL-13, and TNF-α, which have been shown to activate fibroblasts (Van Linthout *et al.*, 2014). Macrophages are the main source of several types of MMPs as well as tissue inhibitors of MMPs (TIMPs). The MMPs mediate not only the degradation of ECM, but they also amplify the inflammatory response and influence the progression of tissue remodeling (Van Linthout *et al.*, 2014; Wynn & Vannella, 2016). Some MMPs are drivers of fibrosis. For example, MMP-12 is a macrophage-secreted elastase that is highly induced by IL-13 in the lung and liver during the development of IL-13-dependent fibrosis (Wynn & Vannella, 2016). Activated macrophages are also able to recruit myofibroblasts and exacerbate inflammatory cell infiltration to sites of tissue injury, leading to profound production of a variety of chemokines, cytokines, and growth factors (Van Linthout *et al.*, 2014). It has been showed that hepatic macrophages enhance myofibroblast survival by stimulating NF-κB activity in fibroblasts, which is critical for the development of liver fibrosis (Wynn & Vannella, 2016).

The contribution of T lymphocytes to organ fibrosis seems to be context dependent. The factors present in the environment trigger specific T-cell populations will subsequently determine the fibrotic outcome (Ueha *et al.,* 2012; Van Linthout *et al.*, 2014). CD4+ T cells are divided into unique subsets on the basis of the cytokines they secrete and their distinct functional abilities (Wynn & Ramalingam, 2012). Th1 effector T cells, defined by their

production of IFN-γ, have anti-fibrotic activity by antagonizing the pro-fibrotic activity of TGF-β1, IL-4- and IL-13 and directly inhibiting fibroblast proliferation (Wynn & Ramalingam, 2012; Van Linthout *et al.*, 2014). Prolonged inflammation seems to induce a shift from a Th1 to Th2 phenotype inducing the infiltration of pro-fibrotic eosinophils via cognate chemokine (e.g., eotaxin) production (Ueha *et al.,* 2012). T-helper type 2 (Th2) cells, characterized by the secretion of cytokines IL-4, IL-5, and IL-13, have been implicated in several experimental and natural models of fibrosis (Wynn & Ramalingam, 2012; Van Linthout *et al.*, 2014). IL-13 can induce fibrosis by stimulating the production and activation of TGF-β and also by directly activating fibroblasts, epithelial cells and smooth-muscle cells (Wynn & Ramalingam, 2012).

CD4+ Th17 cells that express the pro-inflammatory cytokine IL-17A, has also been pointed out in the development and progression of fibrotic disease in lung, heart and liver (Van Linthout *et al.*, 2014). IL-17A seems to promote fibrosis indirectly by inducing tissue damage and inflammation, like persistent neutrophilia that induces apoptosis of vascular endothelial cell. In addition, IL-17A may directly stimulate TGF-β and MMP-1 (Wynn & Ramalingam, 2012). Nevertheless, Th17 cells seem to play a dual role in the fibrosis process, since it has been shown that IL-17A down-regulated the expression of connective tissue growth factor (CTGF) and type I collagen in fibroblasts of healthy patients (Van Linthout *et al.*, 2014). Probably, the presence of regulatory mediators, such as chemokines, transcription factors, and receptors in the particular inflammatory environment, can guide the Th17 response in a pro-fibrotic or anti-fibrotic direction (Van Linthout *et al.*, 2014).

6.2. Molecular activation of myofibroblasts

Fibroblast activation is induced by various stimuli that arise when tissue injury occurs. The expression and synthesis of ECM proteins by the matrix-producing cells is primarily controlled at gene transcription level in response to various extracellular fibrogenic signals. TGF-β1, CTGF and PDGF released from injured epithelial cells and infiltrating cells are pro-fibrotic mediators (Kalluri & Zeisberg, 2006; Liu, 2011; Wynn & Vannella, 2016). In addition, altered MMPs-TIMPs balance and prostaglandins (PG) pathways have also been implicated in fibrogenesis.

6.2.1. Transforming growth factor-β

The classical pro-fibrotic growth factor, which plays a role in fibrogenesis in all organs, is TGF-β. The prominent role of TGF-β in fibrogenesis was first observed when subcutaneous

injection of purified TGF-β induced fibrotic lesions at the injection site. Its action was further corroborated by the observation that neutralization of TGF-β with antiserum ameliorated experimental fibrosis in the kidney, heart and liver (Zeisberg & Kalluri, 2013). TGF-β is overexpressed in all fibrotic tissues, and it induces collagen production in cultured fibroblasts irrespective of their origin (Zeisberg & Kalluri, 2013). However, fibrosis can develop independent from TGF-β, as through an IL-13-mediated pathway (Zeisberg & Kalluri, 2013).

TGF-β plays a central role in fibroblast activation and fibroblast-to-myofibroblast differentiation, and induces the expression of genes for ECM components including collagen type I (Ueha, *et al.*, 2012; Zeisberg & Kalluri, 2013; Seki & Brenner, 2015). However, despite its great potential as a therapeutic target for fibrosis, inhibition of TGF-β signaling has unacceptable side effects due to the critical role of this cytokine in the maintenance of homeostasis (Ueha, *et al.*, 2012). While a key role of TGF-β signaling in organ fibrosis is supported by ample evidence, specific mechanisms involved in deregulation of this pathway towards fibrotic outcomes vary depending on the affected organ (Nakerakanti & Trojanowska, 2012).

TGF-β exerts biological effects by interacting with two types of transmembrane receptors, type I (TGFBR1, also termed Activin Like Kinase 5, ALK5) and type II (TGFBR2) receptors with intrinsic protein serine/threonine kinase activity (Shi & Massague, 2003). The ratio of type I to type II receptor may determine the biological effect of TGF-β on proliferation and target gene expression (Nakerakanti & Trojanowska, 2012). While the TGFBR2 has a high affinity for the ligand, TGFBR1 does not bind to TGF-β. The type II receptor is involved in initial ligand binding. Once the ligand is bound, the type II receptor binds to a type I receptor forming a complex; type I receptor is then phosphorylated by the kinase domain of type II receptor, resulting in a transduction of down-stream signals (Shi & Massague, 2003). Signaling pathways induced by TGF-β1, as well as TGF-β family members, are generally divided into Smad-dependent and Smad-independent ones, like of mitogen-activated protein kinase (MEK/ERK1/2) and phosphatidyl inositol 3 kinase (PI3K) pathways.

Once phosphorylated receptor type I of TGF-β1 is then able to exert its serine-threonine kinase activity phosphorylating Smad2 and Smad3. Receptor type I of bone morphogenetic protein (BMP-7 or ALK3) phosphorylates instead Smad1, Smad5, and Smad8. Upon phosphorylation, they form heterodimers with Smad4, a common mediator of all Smad pathways. The resulting Smad heterocomplexes translocate into the nucleus where they bind directly to DNA and activate specific target genes (Strippoli *et al.*, 2016). TGF-β-mediated Smad2/3 activation induces the transcription of type I and III collagen, promoting fibrosis

(Seki & Brenner; 2015). A third group of Smads composed of Smad6 and Smad7, also called inhibitory Smads, limit BMP-7 and TGF-β1-triggered Smad signaling, respectively (Strippoli *et al.*, 2016).

TGF receptors may also activate signaling pathways independently of Smads. Mitogen activated protein kinases (MAPKs), and PI3Kinase/ Akt (serine-threonine protein kinase, also termed protein kinase B) pathways are relevant in different cellular function elicited by TGF-β1 in different EMT experimental systems (Strippoli *et al.*, 2016). Both Smad and non-Smad pathways converge on activation of Snail, the master factor of EMT (Strippoli *et al.*, 2016).

While many fibrotic disorders are characterized by the elevated levels of TGF-β, additional mechanisms could also account for the increased TGF-β signaling in fibrosis (Nakerakanti & Trojanowska, 2012). As TGF-β is secreted in inactive form, factors that contribute to its activation locally in the affected tissues might play a critical role in perpetuation of the fibrotic process during chronic stages of the disease (Nakerakanti & Trojanowska, 2012). TGF-β is secreted and deposited into ECM as a latent complex (Annes *et al.*, 2003) that is cleaved by MMP-9 and MMP-2 (Nakerakanti & Trojanowska, 2012). In addition, TGF-β1 can directly induce MMP-9 expression via TGF-β receptor (Nakerakanti & Trojanowska, 2012).

Reproductive fibrotic disorders are also characterized by elevated levels of TGF-β. Polycystic ovary syndrome is a reproductive and endocrine disease, characterized by hyperandrogenism, which has been associated with ovarian fibrosis. Dehydroepiandrosterone (a metabolic intermediate in the biosynthesis of androgen)-induced rats exhibited higher levels of fibrosis in both ovarian and uterine tissues mediated by TGF-β1; although it was not determined whether the pathway involved was Smad dependent or Smad independent (Zhang *et al.*, 2013). Aberrant activation of fibrosis may be involved in the pathology of intrauterine adhesions (IUAs), a syndrome of partial or complete obliteration of the uterine cavity in human patients. Expression of TGF-β, CTGF and type I and III collagens was increased in endometrial tissues with IUAs (Hu *et al.*, 2015).

6.2.2. Connective tissue growth factor

Connective tissue growth factor (CTGF) is a potent pro-fibrotic factor that does not behave like a traditional growth factor or cytokine since it does not appear to have a unique receptor to which it binds with high affinity to induce signal transduction (Lipson *et al.*, 2012). CTGF acts by modulating the interaction of cells with the ECM to modify the cellular phenotype (Lipson *et al.*, 2012). CTGF expression is induced by many cytokines and its presence induces formation, activation and deposition of myofibroblasts and remodeling of ECM

proteins. The *CTGF* gene is primarily regulated by TGF-β at transcription level via the Smad pathway, and can act in concert with TGF-β to activate a number of key cell activities (Abraham, 2008). CTGF also induces the expression of a variety of cytokines such as TGF-β and vascular endothelial growth factor (VEGF) which further enhance the expression of CTGF. In the absence of CTGF, fibroblasts are unresponsive to stimuli like TGF-β (Lipson *et al.*, 2012). CTGF has been found over-expressed in several human fibrotic disorders, like liver cirrhosis, keloids and hyperopic scars, diabetic nephropathy, atherosclerosis, cardiac disease, pulmonary hypertension and fibrosis (Abraham, 2008). CTGF also seems to play an important role in arterial aging and vascular fibrosis (Harvey *et al.*, 2016).

6.2.3. Platelet derived growth factor

PDGF signaling is required for many aspects of embryogenesis and regulates the proliferation, migration, survival, and differentiation of mesenchymal progenitor cells that give rise to diverse types of stromal cells in the adult organism (Iwayama *et al.*, 2013).

PDGF is secreted by platelets, monocytes, vascular endothelial cells, and other cell types as a disulfide-linked dimer of two PDGF peptides, giving rise to multiple isoforms including PDGF-AA, BB, AB, CC, and DD (Trojanowska, 2008; Iwayama *et al.*, 2013; Dobie *et al.*, 2015). Mesenchyme-derived cells, for example fibroblasts, smooth muscle cells, and pericytes, are all responsive to PDGF (Iwayama *et al.*, 2013). PDGF signal through two cell surface tyrosine kinase receptors (PDGFRα and PDGFRβ) (Trojanowska, 2008; Iwayama *et al.*, 2013; Dobie *et al.*, 2015). Binding of the ligand to the receptor induces receptor dimerization and downstream signaling through phosphorylation of tyrosine residues on the intracellular domain of the receptor (Trojanowska, 2008; Iwayama *et al.*, 2013; Dobie *et al.*, 2015). PDGF binding induces PDGFR activity, which in turn activates multiple downstream signaling pathways (Iwayama *et al.*, 2013).

Excessive activity of PDGF has been associated with several human disorders, including atherosclerosis, pulmonary hypertension, organ fibrosis and tumorigenesis (Trojanowska, 2008). In severe lung fibrosis induced by irradiation therapy, up-regulation of PDGF (A–D) isoforms expression leading to phosphorylation of PDGF receptor was observed, which was strongly inhibited by PDGFR inhibitors (Abdollahi *et al.*, 2005). In the pathogenesis of systemic sclerosis (SS), pro-fibrotic pathways can be activated by TGF-β and PDGF. In SS fibroblasts, a positive cross-talk between PDGF and TGF-β1 seems to exist. PDGFR expression is up-regulated in response to TGF-β thereby creating an autocrine PDGF-A/ PDGFR loop (Trojanowska, 2008). Also, in dermal fibroblasts, PDGF has been shown to stimulate TGF-β receptors, suggesting an additional reciprocal mode of regulation

(Trojanowska, 2008). PDGF receptors are expressed on adult stem or progenitor cells located in perivascular niches in most mouse organs (Iwayama *et al.*, 2013). Vascular damage is likely an early event in SS, preceding dermal fibrosis and characterized, in the first phases of SS, by perivascular infiltrate of mononuclear immune cells in the vessel wall, obliterative microvascular lesions, and rarefaction of capillaries. Thus, it was proposed that besides directly promoting fibrosis, PDGFR signaling could also be involved in the vasculopathy causing capillary occlusion and regression independently of fibroblast activation (Luchetti *et al.*, 2016). PDGF pathway has also been shown to have profound effects on hepatic fibrogenesis, playing a key role in hepatic stellate cell (HSC) and liver myofibroblast biology (Dobie *et al.*, 2015). In HSC, both PI3K/AKT and ERK/ MAP signaling are central to PDGF-induced proliferation and migration (Dobie *et al.*, 2015).

6.2.4. Matrix metalloproteases (MMP-2 and MMP-9) and tissue inhibitors of metalloproteinase (TIMPs)

The ECM is a multi-molecular complex structure comprising fibers of collagen, elastin, glycoproteins like fibronectin and laminin, and mucopolysaccharides (Harvey *et al.*, 2016). Fibrosis is the result of a disruption of the balance of the ECM, with increased synthesis and deposition of ECM components and decreased degradation of those ECM products. Matrix metalloproteases (MMPs) and TIMPs have been considered as being part of the highly-regulated systems that control ECM turnover.

MMPs are a family of more than 20 metallopeptidases that includes gelatinases, collagenases, stromelysins, matrilysins and membrane-type MMPs (Vandooren *et al.*, 2013). Membrane type MMPs (MT-MMPs) may be regarded as cell receptors for their substrates or inhibitors (Vandooren *et al.*, 2013). MMPs are secreted as zymogens (pro-MMPs) into the extracellular space from a large number of cell types. MMP activity is controlled at three levels: gene transcription, proenzyme activation, and activity inhibition. MMPs transcriptional regulation of gene expression is achieved through different growth factors, cytokines, hormones, and cell-extracellular matrix, and cell-cell interactions. Signaling pathways involved in regulating MMP transcription can enhance or repress MMP expression in a cell type dependent manner (Harvey *et al.*, 2016). MMPs are regulated by MAPKs (Vandooren *et al.*, 2013). Post-transcriptional regulatory processes include mRNA stability, protein translational efficiency, and regulation by microRNAs (Pardo *et al.*, 2016). After translation, zymogen activation of different MMPs may take place intracellularly, at the cell surface, or in the extracellular space. MMPs are activated in the pericellular space by other MMPs, including membrane-type MMPs and MMP-3, or by serine proteases (Harvey *et al.*, 2016). When MMPs are

anchored to the cell membrane they can target their catalytic activity to specific substrates within the pericellular space (Vandooren *et al.*, 2013). Then, the activity of MMPs is inhibited by different mechanisms and molecules, such as TIMPs, α2-macroglobulin, and the membrane associated RECK (reversion-inducing Cys-rich protein with Kazal motifs) (Pardo *et al.*, 2016). TIMPs are specific endogenous inhibitors that bind to the active site of the MMPs blocking access to ECM substrates. Four TIMPs (TIMP-1, -2, -3 and -4) have been identified and are responsible for the inhibition of more than 20 MMPs (Harvey *et al.*, 2016). TIMP-1 is a specific inhibitor for MMP-9 (Vandooren *et al.*, 2013), while TIMP-2 and MMP-14 (also known as MT1-MMP) regulate MMP-2 activity (Giannandrea & Parks, 2014). Although the primary action of TIMPs is to inhibit MMPs, numerous studies have reported cell growth-promoting, anti-apoptotic, steroidogenic and antiangiogenic activities, which are in part independent of MMP inhibition. The main cellular sources of TIMP-1 are macrophages and fibroblasts (Robert *et al.*, 2016).

Regarding the fibrogenic process, the activities of MMPs that can degrade ECM, might be observed to be under-expressed in fibrosis or, if present, could function to resolve the excess matrix. However, some MMPs are indeed anti-fibrotic, whereas others can have pro-fibrotic functions (Giannandrea & Parks, 2014). Although there are many common mechanisms for generating fibrosis among different organs, it is important to note that the roles for specific MMPs are not necessarily the same among different organ systems (Giannandrea & Parks, 2014).

MMP-2 and MMP-9 are enzymes involved in ECM remodeling by degradation of mainly denatured collagens (gelatins) and other matrix-associated substrates such as elastin (Vandooren *et al.*, 2013). Although several studies have investigated the effect of those MMPs on fibrosis development in several organs, obtained results are contradictory with both inhibitory and stimulatory effects being observed. In a study that summarized the effect of MMPs on the development or inhibition of fibrogenic process in different organs, while MMP-9 seems to have a pro-fibrotic effect, MMP-2 anti-fibrotic actions were seen in kidney and liver (Giannandrea & Parks, 2014). It has been reported that MMP-2 participates in the resolution of liver fibrosis by suppressing collagen type I expression instead of its degradation (Duarte *et al.*, 2015). Although MMP-9 expression has been detected in the early stages of hepatic fibrogenesis and it may release/activate TGF-β, MMP-9 may promote hepatic stellate cell apoptosis in the presence of low levels of TIMP-1 (Duarte *et al.*, 2015). Nevertheless, in induced pulmonary fibrosis models, a temporal difference in the expression and localization of MMPs and TIMPs was seen. In the early stages of this disease, MMP-9 activity appears to be predominant, contributing to the disruption of the alveolar epithelial basement membrane

and enhancement of fibroblast invasion to alveolar spaces (Cataldo *et al.*, 2002; Wang *et al.*, 2011). In the late stages of the disease, MMP-2 prevails. *TIMP-1* gene expression is also up-regulated during fibrosis, which might prevent MMP-induced ECM degradation and participate in its accumulation (Cataldo *et al.*, 2002; Wang *et al.*, 2011). The expression of MMP-2 and MMP-9 during the progression of fibrosis suggests that MMP-9 may be linked to inflammation-induced tissue remodeling, while MMP-2 may be associated with impaired tissue remodeling, leading to pathological collagen deposition and interstitial fibrosis (Wang *et al.*, 2011). Also, in idiopathic pulmonary fibrosis (IPF) the synthesis of MMP-2, MMP-8, and MMP-9 may facilitate the trans-endothelial and tissue migration of fibrocytes and also contribute for the remodeling of ECM during the development of IPF (Pardo *et al.*, 2016). Likewise, in vascular fibrosis, MMP2/MMP9 activation through TGF-β1/Smad signaling, induced myofibroblasts activation, collagen production and fibronectin secretion, processes that lead to collagen accumulation in the vascular wall; TGF-β1 further stimulated TIMP, resulting in inhibition of ECM degradation, which further induces ECM accumulation and vascular remodeling and fibrosis (Harvey *et al.*, 2016).

TGF-β and TNF-α induce MMP-9 expression. TGF-β can up-regulate the expression of MMP-9 genes by activating a heteromeric serine/threonine kinase receptor complex, which subsequently activates Smads signaling (Vandooren *et al.*, 2013). Proteolytic activators of MMP-9 include MMP-3, plasmin and neutrophil ELA, among others. In addition, ELA is able to inactivate TIMP-1 when already in complex with MMP-9. This allows pro-MMP-9 to be activated more readily by MMP-3 (Vandooren *et al.*, 2013). MMP-1 and MMP-3 can indirectly induce the expression of MMP-9 in macrophages by triggering the release of TNF-α that induces the expression of PTGS2 and PGE2 secretion. PGE2 subsequently binds to PGE2 receptor 4 (EP4) in the cell membrane and stimulates MMP-9 production through MAPK/ERK1/2 signaling (Vandooren *et al.*, 2013). Regulated expression of MMP-9 by most cell types coincides with the expression of TIMP-1 (Vandooren *et al.*, 2013). It has been speculated that TIMP-1 may be involved in the modulation of inflammatory responses and may also function to stabilize matrix components deposited in the injured lung (Robert *et al.*, 2016).

6.2.5. Prostaglandins pathways in tissue fibrosis

The role of prostaglandins (PG) in promoting human diseases has been widely studied particularly in inflammatory disorders. Recently, additional pathways, other than fibrotic cytokines, eicosanoids such as PGE2 and PGF2α, have been considered in fibrogenesis.

Prostaglandins are lipid autacoids derived from arachidonic acid (AA) that sustain homeostatic functions and mediate several pathogenic mechanisms, including the inflammatory response (Ricciotti & FitzGerald, 2011). Phospholipases A2 (PLA2) enzymes catalyze the release of AA from membrane glycerophospholipids which is sequentially metabolized to prostaglandin G2 (PGG2) and then to PGH2 by prostaglandin-endoperoxide synthase 1 (PTGS1) and/or PTGS2. PTGS1 is constitutively expressed in most tissues and responsible for housekeeping functions, while PTGS2 is regulated by several factors such as cytokines and supports sustained production of PG (Fortier *et al.,* 2008). PGH2 is then converted to various bioactive PGs (thromboxane A2, PGD2, PGE2, PGF2α and PGI2) (Ueno *et al.*, 2005). The PG produced by a given cell largely depends on the expression profile of the individual prostaglandin synthase enzymes (Hata & Breyer, 2004). PGF2α is produced through the reduction of PGH2 by aldoketoreductase 1C synthases (AKR1C3) (Fortier *et al.,* 2008). Three types of PGE synthases (PTGES) controlling PGE2 production in cells have been identified. Two are membrane-associated (mPTGES-1 and mPTGES-2) and the third is cytosolic (cPTGES). mPTGES-2 is constitutively expressed in many tissues, whereas mPTGES-1 expression is responsible for sustained production of PGE2 (Fortier *et al.*, 2008) and is induced in response to inflammation (Stichtenoth *et al.*, 2001). PG are ubiquitously produced and act locally in an autocrine or juxtacrine manner, through specific receptors, due to their extremely short half-life in blood (Hata & Breyer, 2004; Oga *et al.*, 2009). They elicit a diverse set of effects that modulate many physiological systems, like reproductive ones, but also has been implicated in a broad array of diseases (Hata & Breyer, 2004). Activation of a given prostaglandin receptor by its associated ligand may elicit various responses in different cell types and tissues.

PGF2α acting on its receptor (FP) was recently discovered to enable pulmonary fibrosis (Oga *et al.*, 2009). Increased collagen I (COL1) and III (COL3) through a FP receptor/protein kinase C (PKC)/Rho kinase cascade was depicted not only in cardiac fibroblasts (Ding *et al.*, 2012), but also in an *in vivo* animal model where myocardial fibrosis was induced (Ding *et al.*, 2014). In a bleomycin-induced pulmonary fibrosis model, plasma PGF2α metabolites were associated to disease severity and prognosis (Aihara *et al.*, 2013). In the lungs, PGF2α – FP appears to act on fibrosis but not on inflammation (Oga *et al.,* 2013). Nevertheless, whether TGFβ-1 and PGF2α stimulate fibrogenesis through independent pathways has been questioned, and differs depending on study models. Some authors have referred that PGF2α stimulates TGF-β production (Hou *et al.*, 2008) and plays a central role in the establishment of fibrosis (Oga *et al.*, 2009). In fibroblasts from mice with dermal sclerosis, alpha2-antiplasmin (2AP), a serine protease inhibitor (serpin) elevated in fibrotic diseases, induced

PGF2α synthesis that in turn promoted TGFβ-1 production and fibrosis (Kanno *et al.*, 2013). However, in contradictory studies, PGF2α, which was abundant in broncho-alveolar lavage fluid of mice with induced pulmonary fibrosis, stimulated proliferation of lung fibroblasts and collagen production via PGF2α receptor (FP) and independently of TGF-β (Oga *et al.*, 2009). Using a mice model lacking each prostaglandin receptor, Oga and co-authors (2009) showed that loss of FP selectively attenuated pulmonary fibrosis, without interfering with phosphorylation of Smad2. Deficiency of FP and inhibition of TGF-β signaling additionally decrease fibrosis (Oga *et al.*, 2009). PGF2α mobilizes signaling to induce collagen production through Rho kinase pathway (Oga *et al.*, 2013). Besides, in primary rat cardiac fibroblasts, treatment with PGF2α increased COL1 and COL3 expression by activating the FP receptor/PKC/Rho kinase cascade independent of TGF-β1, and downregulating the PI3K/Akt pathway (Ding *et al.*, 2012). Also, synovial fibrosis establishment was positively associated with PGF2α levels, while inhibition of TGFβ-1 signaling had no effect on the pro-fibrotic changes (Bastiaansen-Jenniskens *et al.,* 2013). Thus, in some systems, PGF2α-FP signaling facilitates fibrosis independently of TGF-β1 (Oga *et al.*, 2009; Olman, 2009; Ding *et al.*, 2012; Bastiaansen-Jenniskens *et al.,* 2013).

Several biological effects in many organs have been ascribed to PGE2, such as pro- and anti-inflammatory actions, which depend on the activation of its four subtypes prostanoid receptors (EP1, EP2, EP3 or EP4) (Kalinski, 2012). Signaling through EP2 and EP4 mediates the dominant aspects of the PGE2 anti-inflammatory and suppressive activities (Kalinski, 2012). In addition, PGE2 is known to be an anti-fibrotic mediator acting as an autocrine factor and controlling cellular over-activation (Sokolova *et al.*, 2005). As an anti-fibrotic mediator, PGE2 protects different tissues by inducing fibroblast apoptosis (Huang *et al.*, 2009), decreasing fibroblast proliferation, migration and differentiation into myofibroblasts and reducing collagen production and deposition by inhibition of its mRNA synthesis (Sokolova *et al.*, 2005; Stratton & Shiwen 2010; Ueha *et al.*, 2012). One putative mechanism by which PGE2 may exert its antifibrotic effects is by inhibiting TGF-β–induced myofibroblast differentiation (Bozyk & Moore, 2011). PGE2 ability to alter cytoskeletal architecture and disrupt the formation of focal adhesions, by activating PTEN (phosphatase and tensin homolog deleted on chromosome 10), may also limit myofibroblast differentiation (Bozyk & Moore, 2011). Previous studies using the bleomycin model of pulmonary fibrosis have shown the protective effects of PGE2 known to act through cAMP/ protein kinase A (PKA) signaling (Dackor *et al.*, 2011). In a model of bleomycin-pulmonary fibrosis, anti-fibrotic effect of noscapine (an alkaloid used as an antitussive medication in humans) was related with a rapid and profound activation of cAMP/PKA signaling mediated by EP2 in pulmonary fibroblasts

(Kach *et al.*, 2014). Furthermore, PGE_2 had no therapeutic effect on these parameters when administered 14 days after bleomycin challenge (Kach *et al.*, 2014).

In fibroblasts from patients with severe fibrosing systemic sclerosis, levels of mPTGES and PGE2 were greatly elevated compared to healthy controls (Stratton & Shiwen, 2010). Likewise, mPTGES-1 protein expression was elevated in systemic sclerosis skin fibroblasts and in bleomycin-exposed mice (McCann *et al.*, 2011). In addition, mPTGES-1-null mice were resistant to bleomycin-induced inflammation, collagen production and myofibroblast formation (McCann *et al.*, 2011). Nevertheless, diminished PGE2 levels in patients with pulmonary fibrosis and reduced synthesis of PGE2 from fibroblasts obtained from those patients were also reported (Moore *et al.*, 2000; Sokolova *et al.*, 2005). Consistent with the notion that inflammation is involved with the onset of fibrosis, it was suggested that early PGE2 release following tissue injury promotes inflammatory cell infiltration (Stratton & Shiwen, 2010; McCann *et al.*, 2011). This in turn enhances ECM gene induction, via the release of TGF-β or PDGF by inflammatory cells, which may cause secondary enhancement of fibroblast activation (Stratton & Shiwen, 2010).

Anti-fibrotic action of PGE2 has also been linked to EP2 or EP4 signaling, although the subtype of receptor that is involved on this action seems to be tissue-dependent. In lung fibrosis EP2 appear to be responsible for PGE2 anti-fibrotic effects (Huang *et al.*, 2010; Kach, *et al.* 2014; Wei *et al.*, 2014). However, endogenous PGE2-EP4 signaling appears to have protective roles against cardiac fibrosis (Ishiwata, *et al.*, 2013; Harada *et al.*, 2017; Wang, *et al.* 2017) and renal fibrosis (Nakagawa, *et al.* 2012). Nevertheless, in chronic kidney failure both EP2 and EP4 receptors seem to be equally important in preserving the progression of fibrosis (Xi *et al.*, 2016). Besides being tissue-dependent, the functions of these receptors also seem to be context dependent, since increased-EP4 fibrotic effects have been reported. In fact, chronic administration of EP4-selective agonist exacerbated albuminuria and fibrosis of the kidney in an animal model of type 1 diabetes, through IL-6 (Mohamed *et al.*, 2013). Diabetes-induced expression of inflammatory cytokines (TNF-α or TGF-β), COL1, CTGF and IL-6 was enhanced in EP4 agonist-treated mice. Nevertheless, the EP4 agonist used did not alter macrophage infiltration. So PGE2–EP4 pathways may play an active role in the progression of renal tubulointerstitial fibrosis via EP4 (Mohamed *et al.*, 2013). It has been proposed that high level of IL-1 acts to induce PGE2 production in human lung fibroblasts through PTGS2 induction and that fibroblast proliferation inhibitory activity of PGE2 is biphasic. EP2 signaling mediates the proliferative suppressive effects of high concentrations of PGE2, while EP3 signaling reduction mediates the proliferative stimulatory effects of mid-range concentrations of PGE2 (White *et al.*, 2008).

Several conditions that prime the suppression of different PGE2 mediators in the tissues, by preventing them from exerting their anti-fibrotic functions, may trigger fibrogenesis. Besides impaired PGE2 synthesis, decreased PTGS2 or mPTGES-1 or deficiency of EP2 have been shown to promote fibrogenesis. The ability of fibrotic cells to release PGE2 upon thrombin stimulation was significantly impaired compared with normal cells (Sokolova *et al.*, 2005). A similar reduced capacity of PGE2 synthesis in fibrotic lung fibroblasts was reported for other stimuli, in particular for TGF-β1, phorbol myristate acetate (PMA), lipopolysaccharide (LPS), and IL-1, due to the diminished ability of fibrotic cells to upregulate PTGS2 mRNA level (Sokolova *et al.*, 2005). Impaired production of PGE2 was responsible for enhanced fibrogenesis in bleomycin-induced pulmonary fibrosis in granulocyte-macrophage colony-stimulating factor (GM-CSF) deficient mice. Although GM-CSF is a cytokine that regulates the proliferation and differentiation of cells in the granulocyte-macrophage lineage, enhanced fibrogenesis was not related to increased recruitment of inflammatory cells (Moore *et al.*, 2000). In addition, mice deficient in mPTGES-1 exhibited more severe fibrotic lesions and a decrease in PGE2 content in lungs after bleomycin treatment (Wei *et al.*, 2014). In cultured human lung fibroblast cells, siRNA-mediated knockdown of mPTGES-1 augmented TGF-β1 and α-SMA protein expression, which was reversed by treatment with PGE2, selective EP2 agonist and focal adhesion kinase (FAK) inhibitor (Wei *et al.*, 2014). Activation of mPTGES-1-PGE2-EP2 signaling pathway also seems to exert an essential effect against kidney fibrosis (Jia, 2015). Besides, patients with idiopathic pulmonary fibrosis (IPF) and fibroblasts obtained from those patients showed diminished PGE2 synthesis. Yet, those fibroblasts and mice with induced fibrosis did not respond to exogenous PGE2 treatment. Down-regulation of EP2 and consequent PGE2 resistance in fibroblasts from both mice and humans with pulmonary fibrosis were both mediated by DNA hypermethylation; treatment with DNA methylation inhibitors decreased EP2 methylation, increased EP2 mRNA and protein expression levels, and restored PGE2 responsiveness in fibrotic fibroblasts (Huang *et al.*, 2010). Also, increased ECM stiffness has been linked to the suppression of lung fibroblast expression of PTGS2 and synthesis of PGE2 (Liu *et al.*, 2010).

Three recent reports recognized PGE2 as an endogenous physiological inhibitor of NETosis through EP2 or EP4-signaling. PGE2 clearly reduced NETs formation and directly suppressed PMN phagocytosis (Marey *et al.*, 2014). Human patients that highly expressed PGE2 after hematopoietic stem cell transplantation have enhanced susceptibility to bacterial pathogens and reduced NETosis. Administration of PGE2 resulted in reduced NETosis *in vitro* and in a mouse model (Shishikura *et al.,* 2015). It was proposed that inhibition of NETs by PGE2 signaling is a universal feature of PMN, since this effect has been observed with PMN

obtained from different organic compartments and animal species. Also, PGE2 was able to inhibit NETosis induced by a variety of stimuli (Domingo-Gonzalez *et al.*, 2016). So, this novel role for PGE2 as an inhibitor of NETosis may also be a mechanism of protection against the development of chronic diseases. By preventing the uncontrolled release of NETs, it prevents perpetuation of stimuli that can trigger a chronic inflammatory process and consequent fibrosis. Therefore, this role of PGE2 appears to be directly related to its EP2 or EP4 mediated anti-inflammatory and anti-fibrotic effect.

6.3. Molecular mechanisms involved in the persistence of fibroblast-activated state in fibrotic tissues

In tissue fibrosis, fibroblasts maintain their activated state, often until organ death (Kalluri & Zeisberg, 2006). The exact molecular mechanisms behind the persistence of this activated-state fibroblasts in fibrotic tissues, even when the initial insult has regressed are still unknown. The continuous secretion of ECM constituents, growth factors and cytokines by activated fibroblasts results in a self-perpetuating autocrine loop, which stimulates other fibroblasts and prevents resolution of the initial injury (Kalluri & Zeisberg, 2006). Activated fibroblasts release chemokines that promote the recruitment and activation of circulating leucocytes that produce MMP-9, allowing degradation of the basal membrane and subsequent trans-endothelial migration (Van Linthout *et al.*, 2014). In a recent study, it was suggested that although TGF-β1 is a critical cytokine in the process of trans-differentiation of proximal tubular cells (PTC) in interstitial myofibroblasts, other factors are likely to act in a cooperative way to complete the process (Yen *et al.*, 2016). While TGF-β1 alone caused incomplete and reversible transdifferentiation of PTC, combination of TGF-β1 stimulation and contact with type I collagen substratum cooperatively caused complete and irreversible PTC transdifferentiation; also, a time and dose-dependent induction of MMP-9 in PTC was seen (Yen *et al.*, 2016). Disruption of the tubular basement membrane by MMP-9 and contact of PTC with interstitial components appear to be essential to the TGF-β1-induction of epithelial-mesenchymal transition. It was speculated that transdifferentiated PTC once moved into the interstitium, attached in a microenvironment rich in type I collagen, causes an irreversible phenotypic alteration (Yen *et al.*, 2016). Also, in lung fibrotic tissue, increased tissue mechanical stiffness due to ECM components deposition might play a critical role in fibroblast activation that promotes and amplifies progressive fibrosis (Liu *et al.*, 2010). This self-sustained fibroblast proliferation and ECM synthesis response to increasing ECM stiffness was due to the suppression of PTGS2 expression and PGE2 synthesis (Liu *et al.*,

2010). Fibroblasts within a stiffened fibrotic lesion were primed to engage in further fibrogenic activities (Liu *et al.*, 2010). Since similar stiffness levels and cellular behaviors are observed during fibrogenesis in other soft tissues, it was proposed that the mechanobiological feedback loop detected in lung fibrotic tissue may represent a common feature of progressive fibrosis (Liu *et al.*, 2010). In the early stage of renal fibrosis, the collagen matrix is susceptible to proteolysis, and therefore makes fibrosis potentially reversible, leading to wound healing (Liu, 2011). However, biochemical modifications of the ECM proteins by cross-linking, induced by enzymes such as tissue transglutaminase and lysyl oxidase, render them stiff and resistant to proteolysis, as fibrosis progresses (Liu, 2011).

It was suggested that disease persistence brings about lasting changes to myofibroblasts- a form of reprogramming (Duffield, 2014). Studies of liver disease suggest the equivalent cellular lineage, such as hepatic stellate cells, can revert to pericytes following resolution of chronic disease *in vivo*, but are persistently reprogrammed to respond more vigorously to a new injury (Kisseleva *et al.*, 2012). Epigenetics may also be an explanation for distinct risks for developing fibrosis (Zeisberg & Kalluri, 2013). Studies on reprogramming of myofibroblasts have focused on DNA methylation patterns in human cells grown from diseased *versus* normal kidney (Bechtel *et al.*, 2010). Fibroblasts from diseased kidney show distinct methylation patterns that result in silencing of the RAS intracellular signaling pathway and myofibroblast persistence (Bechtel *et al.*, 2010). Chronic stimulation of fibroblasts with pro-fibrotic cytokines triggers epigenetic modifications in fibroblasts phenotype, like aberrant promoter methylation of specific genes, leads to silencing of genes and to the maintenance of an activated phenotype. This endogenous pro-fibrotic mechanism exacerbation has been described in systemic sclerosis and in liver, lungs and heart fibrosis (Bechtel *et. al.*, 2010; Huang *et. al.*, 2010; Watson *et. al.,* 2014; Bergmann & Distler, 2017).

7. Mare endometrial fibrosis

Endometrosis, chronic degenerative endometritis, endometrial fibrosis and chronic degenerative endometrial disease are common names for the same nosological entity. It is a severe and complex age-related fibrotic disease (Hoffmann *et al*., 2009a). Endometrosis is characterized by stromal endometrial fibrosis accompanied by periglandular fibrosis, sometimes with pathologic nesting of gland branches within a fibrotic matrix around periglandular branching points. Lymphatic lacunae and reduction in the number of uterine glands and atrophy can also be observed (Kenney *et al*., 1978). Endometrial vascular alterations, such as angiosis and elastofibrosis of arteries, veins and lymphatic vessels in the

uterine wall are also seen (Oikawa *et al*., 1993; Snider *et al*., 2011; Hanada *et al*., 2014). The incidence and severity of the angiopathies increase as mares age (Schoon *et al*., 1999; Hanada *et al*., 2014). Degenerative processes, such as elastosis, lymphangiectasia, excessive exudate, loss of epithelium and epithelial hyperplasia, will interfere with mare's ability to become pregnant (LeBlanc & Causey, 2009).

For the last 40 years, endometrial biopsy has been the main procedure for histopathologic assessment of equine uterine health (Kenney *et al*., 1978; Snider *et al*., 2011). A scoring system was developed to evaluate endometrial health based on the quality of glands and lymphatics, fibrotic changes, and infiltration of inflammatory cells. This score is associated with the prognosis of carrying a foal to term (Kenney & Doig, 1986). Mares were classified into 4 categories according to the acute and chronic changes observed in the histopathology of the endometrium (Kenney & Doig, 1986). An endometrium graded as category I is considered normal and healthy, with active, evenly distributed uterine glands and little to no inflammatory cells. In category IIA are included endometria with mild, scattered inflammation and mild fibrosis, while category IIB corresponds to moderate scattered inflammation and moderate fibrosis. In contrast, dilated glands surrounded by layers of fibrotic cells are inactive and considered to impair uterine function. They are assigned to the most severe score of category III (Kenney & Doig, 1986). Limitations of Kenney-Doig system relate to its disregard of important histological findings, such as angioses and endometrial maldifferentiation (Snider *et al*., 2011).

More recently, an additional system for endometrial evaluation has been proposed, that divides the equine endometrosis in a non-destructive (characterized by mild changes in the glandular epithelium) and a destructive form (Hoffmann *et al*., 2009a). In the destructive form, glands show signs of extensive epithelial degeneration with loss of normal glandular architecture and invasion of the glandular lumen by the stromal cells (Hoffmann *et al*., 2009a). Based on the morphology of the stromal cells, an active or inactive state can be distinguished in fibrotic foci (Hoffmann *et al*., 2009a). Active endometrosis contains metabolically active stromal cells with active collagen synthesis and ECM deposition, whereas inactive endometrosis involves metabolically inactive stromal cells. Hoffmann *et al*. (2009a), based on morphological/immunohistochemical findings during histological evaluation, proposed that the pathogenesis of endometrosis, involves an active fibrosis, destructive active fibrosis, inactive fibrosis and destructive inactive fibrosis. Those entities represent different evolution phases of the fibrotic process, which are independent of uterine control mechanisms (Hoffmann *et al*., 2009a). In fibrosis, the main source of collagen are

activated fibroblasts. These α-SMA-myofibroblast cells have been characterized in altered horse endometrium.

The hallmark histopathologic feature of equine endometrosis is endometrial periglandular fibrosis (EPF), where lymphocytes, plasma cells and macrophages infiltrate the endometrium (Granjam & Evan, 2006). Morphologically, EPF consists of concentric layers of transformed fibroblasts with myofibroblastic features and deposition of fibrillar collagen around unaltered glandular basal laminae (Evans *et al*., 1998). Using picrosirius red-stain, endometrial periglandular collagen volume fraction values varied among the different Kenney endometrial categories and increased with aging (Evans *et al*., 1998). Also in mare endometrial fibrotic tissue, periglandular stromal cells, expressing α-SMA were characterized immunohistochemically as myofibroblasts, with collagen IV, laminin and fibronectin deposition outside the basement membrane (Walter *et al*., 2001). Category IIB endometria exhibit an ongoing active myofibroblastic transformation that is absent in category I endometrium and is arrested in category III. This myofibroblastic arrest was related to the expansion of the ECM (Granjam & Evan, 2006). According to Hoffmann and co-authors (2009a), destructive endometrosis is characterized by a large number of α-SMA myofibroblasts, pronounced epithelial expression of vimentin, excessive accumulation of ECM, glands alterations (cystic dilations and glands destruction) and alteration of the basal lamina. Large discontinuities of the glandular basal lamina are predominantly seen in destructive endometrosis (Hoffmann *et al*., 2009a). Also, in all mares with endometrosis, α-SMA protein expression was detected in fibroblasts surrounding fibrotic uterine glands and a high discontinuity of epithelial basal lamina was noted (Mambelli *et al*., 2014).

Although α-SMA myofibroblasts responsible for ECM deposition have been identified within fibrotic foci, studies concerning the type of collagen are in part, contradictory. In endometrial fibrotic tissue, no alterations in collagens type I and III have been referred (Walter *et al*., 2001). Likewise, regarding the collagen type, no differences were found among the affected endometrial fibrosis areas in all the endometrial tissues (Lunelli *et al*., 2013). All samples had a predominance of type III collagen, although none of those samples were in category III (Lunelli *et al*., 2013). Nevertheless, in an EPF morphometric analysis using picrosirius red stained endometrial tissues, deposition of type III and type I collagen was seen in severe endometrosis, with collagen type I predominance (Porto, 2006). Additional studies that evaluated the endometrial fibrosis in mares, considering histomorphological, histochemical and immunohistochemical characteristics of active fibrosis, destructive active fibrosis, inactive fibrosis and destructive inactive fibrosis, showed that the arrangement of collagen fibers and their characteristics were related to the evolution of the process (Masseno, 2012;

Costa 2015). While in both active and destructive active endometrosis a less dense reticular collagen arrangement predominates, in inactive and inactive destructive endometrosis, a denser collagen prevails. Samples of active destructive endometrosis presented greater expression of α-SMA, while weaker expression of α-SMA in fibrotic nests of inactive endometrosis reflects the predominance of fibrocytes in these lesions (Masseno, 2012). A parallel study with immunohistochemical and picrosirius red stained endometrial biopsies revealed that the dense collagen fibers that correspond to type I collagen were prevalent in inactive and inactive destructive endometrosis, while the loose collagen fibers correspond to type III collagen and were predominant in active and active destructive endometrosis (Costa, 2015). In the same study, a greater amount of periglandular myofibroblasts were observed in active and in active destructive processes, compared to inactive and inactive destructive endometrosis (Costa, 2015). It was suggested that the presence of these cells in active processes were strongly related to an increased deposition of collagen type III in fibrotic nests (Costa, 2015).

Concerning glandular estrogen (ESR) and progesterone receptors (PGR) expression within the fibrotic tissues, Aupperle and collaborators (2000) referred that expression of those receptors in the fibrotic glands were lower when compared with non-fibrotic areas. A cycle-asynchronous staining for ESR and PGR in the stromal cells in the areas of periglandular fibrosis and in the glandular epithelia was noted; the affected areas appear to become independent of the endocrine uterine control mechanisms (Hoffmann *et al*., 2009a; Lehmann *et al*., 2011). A mild increase of epithelial ESR and PGR was seen in active non-destructive fibrotic foci, while all other types of endometria showed a decrease in their expression, mainly in severe destructive fibrosis (Hoffmann *et al*., 2009a). Likewise, the majority of barren (showing destructive mostly moderate type of endometrosis) and foaling (exhibiting mild, nondestructive endometrosis) mares had a markedly reduced fibrotic stromal cell expression of ESR and PGR compared with the unaltered stroma. Epithelial ESR and PGR expression was mildly increased in active nondestructive fibrotic foci and mildly decreased in inactive nondestructive endometrosis, both in barren and foaling mares (Lehmann *et al*., 2011). However, contradictory results indicated no differences in ESR and PGR expression in the endometrium with fibrosis, although in this study there were no type III endometrial samples (Lunelli *et al*., 2013).

Glandular basal lamina damage seems to be crucial to tissue fibrosis response. Deposition of collagen type IV, laminin and fibronectin outside the basement membrane with large discontinuities of the glandular basal lamina were predominantly seen in destructive endometrosis (Walter *et al*., 2001; Hoffmann *et al*., 2009a). In another study, thickness of

basal membrane, but not related to increased expression of type IV collagen was observed in the active and active destructive endometrosis, while active destructive and inactive destructive endometrosis exhibited disruption areas in type IV collagen fibers (Costa, 2015). It was suggested that paracrine mechanisms and severe damage of the basal lamina may explain the cycle asynchronous pattern of the glandular epithelia within fibrotic foci, since the effect of the steroid hormones on the glandular epithelia is physiologically mediated by the adjacent stromal cells (Hoffmann *et al*., 2009a). Direct stromal-epithelial contact, as well as interaction between epithelial cell surface integrins and the fibrotic ECM may alter the complex paracrine interactions between the epithelia and the underlying stroma (Hoffmann *et al*., 2009a). Thickening of the basal membrane in active and destructive active endometrosis, sometimes with its disruption was also noted by Hanada *et al*. (2014). It was postulated that the presence of degenerated or necrotic epithelial cells in periglandular fibrosis was mainly due to damage to the basal membrane. This allows a direct contact between stromal and epithelial cells, as well as the interactions between fibrotic ECM and glandular epithelial cells (Hanada *et al*., 2014).

Local hypoxia is discussed in the pathogenesis of endometrosis as a result of perfusion disorders of the endometrium in the context of degenerative vessel alterations (Grüninger *et al*., 1998; Hoffmann *et al*., 2006, Hoffmann *et al*., 2009a; Masseno, 2012; Hanada *et al*., 2014). Angiosclerosis refers to thickening of vessel walls due to degenerative changes leading to reduced elasticity of the walls and lower perfusion. In older maiden mares, vessels were frequently affected by angiosclerotic changes and the incidence and severity of angiosis increased with the number of previous pregnancies and with advancing age (Grüninger *et al*., 1998). The results of Hoffmann *et al*., (2006) showed that two thirds of the mares with endometrosis also have angiosclerosis. Contradictory results reported that although large percentage of both barren and foaling mares has angiosclerosis, no correlation was found between endometrosis and angiosclerosis (Lehmann *et al*., 2011). Yet, as fibrotic lesions of inactive and inactive destructive endometrosis were related to severe vascular fibrotic lesions it was suggested that they trigger endometrosis (Masseno, 2012). It was also proposed that mare aging could play a role in the pathogenesis of endometrosis, since elastofibrosis of arteries, veins and lymphatic vessels in the uterine wall increased with advancing age (Hanada *et al*., 2014). Circulatory disturbances due to intrauterine angiosis or angiopathy, particularly the reduction of the arterial blood supply and disturbance of venous drainage, resulting in a reduction of lymphatic drainage (lymphatic edema) may be related to the onset and progression of endometrial fibrosis (Hanada *et al*., 2014). Indeed, decreased uterine blood flow in older mares with higher Kenney and Doig endometrial categories and impaired

uterine vascular perfusion has been found in early pregnant mares with endometrial degeneration (Ferreira *et al*., 2015).

7.1. Endometrial secretory alterations during the course of endometrosis

An altered uterine secretion pattern in endometrial fibrotic tissues seems to be due to a different paracrine stimulation of the glands by adjacent fibrotic stromal cells, impaired hormonal control and/or progressive epithelial destruction (Hoffmann *et al*., 2009b; Lehmann *et al*., 2011). In Hoffmann *et al*., (2009b) work, impaired calbindin, uterocalin (transport protein which ensures the nutrient supply for the conceptus during the preimplantation period) and glycogen by fibrotic tissues was noted. This may lead to an insufficient nutritional supply of the early conceptus with its subsequent death. Besides, decreased uteroglobin secretion within fibrotic foci was also referred (Hoffmann *et al*., 2009b). In another study, barren mares showing a destructive mostly moderate type of endometrosis, uteroglobin was decreased in affected glands, while an increased expression pattern was noted in foaling mares exhibiting a nondestructive endometrosis (Lehmann *et al*., 2011). It was suggested that impaired uteroglobin may support the development of fibrosis by enhancing ECM deposition (Hoffmann *et al*., 2009b; Lehmann *et al*., 2011). In addition, modified uteroglobin secretion pattern, by inhibiting phospholipase A2, a key enzyme in prostaglandin (PG) metabolism, may be involved in altered PG synthesis in fibrotic tissues (Hoffmann *et al*., 2009b; Szóstek *et al*., 2012). An independence of glandular fibrotic nests from the ovarian-uterine control mechanisms, exhibiting specific differentiation dynamics was proposed as a possible cause of the altered mRNA transcription of PG synthases and PG concentration during the course of endometrial fibrosis development (Szóstek *et al*., 2012). Basal secretion of PGE2 and PGF2α from incubated category II endometria was higher compared to the secretion of both PG from category I tissues, while it was decreased in category III endometrium compared to category I (Szóstek *et al*., 2013). In addition, the highest concentration of PGF2α was found in healthy endometria of mares in the follicular phase, with decreased PGF2α production in categories II and III endometria in both follicular and mid-luteal phases (Szóstek *et al*., 2012). In contrast, no changes in the secretion profile of PGE2 and PGF2α during the course of fibrosis was also reported (Gajos *et al.,* 2015). In that same study, increased PGE2 production was only noted in mares suffering from subclinical endometritis with accompanying fibrosis (Gajos *et al.,* 2015).

7.2. Physiopathological mechanisms involved in endometrosis

The physiopathological mechanisms leading to the endometrial periglandular fibrosis (EPF) in the mare are still not completed known. Different growth factors and cytokines, especially TGF-β1, might be involved in the differentiation of endometrial stromal cells into myofibroblasts in mare endometrium. In a study examining equine endometrial mRNA expression of *TGF-β1* and *TNF-α*, no differences were found in the expression of this genes between endometrial categories (Cadario *et al*., 2002). Nevertheless, TGF-β1 or TNF-α proteins and their receptors were not evaluated. Granjam & Evans (2006) studying the involvement of endometrial TGF-β1 and angiotensin-converting enzyme (ACE) during the progression of equine periglandular fibrosis found that both TGF-β1 and ACE were present in macrophages, endothelium (during angiogenesis), and myofibroblasts at sites of fibrosis. The concentration of TGF-β1 increased proportionally with the degree of severity of endometrial fibrosis, suggesting its involvement in the phenotypic transformation of fibroblasts into α-SMA myofibroblasts (Granjam & Evans, 2006). Angiotensin converting enzyme is involved in the formation of angiotensin II, a pro-hypertensive vasoactive agent (Harvey *et al*., 2016). It also has an important fibrogenic role by increasing fibrillary collagen synthesis and reducing collagenase activity in human hypertensive heart disease (Harvey *et al*., 2016). It acts by stimulating pro-fibrotic signaling cascades including p38 mitogen-activated protein kinases (p38 MAPK) and the TGF-β1/SMAD pathway (Harvey *et al*., 2016). Since endometrial ACE-binding activity was consistently higher in equine category IIB endometria, it was proposed that locally generated angiotensin II initiates the expression of TGF-β1 resulting in myofibroblastic transformation (Granjam & Evan, 2006). In contrast, reduced expression of both TGF-α or TGF-β isoforms in stromal cells within the different endometrial foci was noted (Kiesow *et al*., 2011). Disturbed hormonal stimulation or stromal synthesis disorders due to altered epithelial/stromal interactions were identified as possible causes for the decreased TGFs expression (Kiesow *et al*., 2011). In a study carried out in our laboratory the expression of TGF-β1 and its receptors in category IIB or III mare endometrial tissues was evaluated by immunohistochemistry (Cardoso, 2015). Expression of TGF-β1 and its receptors was noted in all mares. TGF-β1 and TGFBR1 showed a similar expression pattern and were mainly found in the glandular epithelium, endothelium and macrophages. Higher expression of TGF-β1 and TGFBR1 in the glandular epithelium of dilated glands or in the glands located in fibrotic foci was noted. TGFBR2 showed a different expression pattern, as no expression of this receptor was found in endothelial or in glandular epithelial cells. Its expression was more sporadic and found mainly in stromal cells involving dilated glands or glandular nests, ciliated

cells and macrophages (Cardoso, 2015). Besides, endometrial *TGFBR1* and *TGFBR2* genes transcription was lower in equine category I/IIA endometria, while increased *TGFBR1:TGFBR2* mRNA ratio in IIB/III tissues were detected (Morazzo, 2017; Morazzo *et al*., 2017). So, the expression of TGF-β1 and its receptors in fibrotic endometrial tissues suggests a role of this pro-fibrotic cytokine in the progression of mare endometrosis.

Nodal is a member of the transforming growth factor-beta (TGF-β) superfamily. The Nodal/ Lefty (antagonist that blocks Nodal receptor complex) pathway was shown to be involved in endometrial regulation of ECM and connective tissue during the menstrual cycle and Nodal activity was upregulated in endometrial carcinomas tissues that lacks Lefty (Papageorgiou *et al*., 2009). Presence of Nodal in the *corpus luteum* of the mare was related to the mechanism responsible for functional luteolysis, decreasing PGE2 and increasing PGF2α (Galvão *et al*., 2016). The presence of Nodal and its relationship with fibrogenesis was most recently evaluated in mare endometrium (Volpi *et al*., 2014; Morazzo, 2017; Morazzo *et al*., 2017). Nodal and its receptors ALK-4 and ALK-7 proteins were expressed in superficial and glandular epithelium, with lower staining of ALK-7, regardless of estrous phase and endometrium category (Volpi *et al*., 2014). In addition, mRNA transcription of Nodal and its receptors was lower in categories I and IIA, compared to categories with strong fibrosis (IIB or III) (Volpi *et al*., 2014). When Nodal was used in culture, it was able to enhance mRNA expression of TGF-β1 receptors, *TGFBRI* and *TGFBRII*, in category I/IIA endometria (Morazzo, 2017). Nodal was also able to alter prostaglandin pathways. It inhibited PGE2 production in follicular phase and gene transcription of its *EP2* and *EP4* receptors in category I/IIA endometria (Morazzo, 2017), both conditions linked to impaired anti-fibrotic action of PGE2 in other tissues (Bozyk & Moore, 2011). In addition, the production of $PGF_{2\alpha}$ increased in mid-luteal phase endometrial tissues with Nodal stimulation (Morazzo, 2017), which is related to a pro-fibrotic effect in other organs, like lung (Oga *et al*., 2009).

Since in endometrial fibrosis ECM components deposition occurs, the involvement of ECM degrading endopeptidases known as MMPs and their inhibitors (TIMPs) has also been addressed. During the normal equine estrous cycle, secretion of MMP-2 and MMP-9 into the uterine lumen is minimal, although increased inhibitory activity is seen by TIMP-2 during diestrus (Oddsdóttir, 2007). Results concerning MMPs in fibrotic endometria are in part contradictory. Walter *et al*. (2005), reported that overall MMP-2 expression increased in severely affected endometrial tissues mainly in diestrus. Likewise, a more intense immune reaction for MMP-2 in glandular epithelial cells of category III tissues, but a decreased MMP-9 in vascular walls in fibrotic tissues were observed (Porto *et al*., 2011). It was proposed that this decreased expression of MMP-9 in vascular walls could be related to impaired

angiogenesis, which would lead to a greater accumulation of fibrotic tissue (Porto *et al*., 2011). In another study MMP-2 was also increased in stromal cells of all endometrial foci, and it was hypothesized this could be responsible for the progressive destruction of the glandular basal lamina (Kiesow *et al*., 2011). Also, we have shown that in mid-luteal phase endometrial explants, PMN elastase, increased active MMP-9 expression after 24h incubation, and pro-MMP-2 after 48h (Rebordão *et al*., 2015). The results of these studies are consistent with those described in lung fibrosis establishment. In the early stages of this disease, the activity of MMP-9 appears to be predominant, and likely contributes to the disruption of the alveolar epithelial basement membrane allowing fibroblast invasion of alveolar spaces (Wang *et al*., 2011). In the late stages of the disease, MMP-2 appears to become predominant. *TIMP-1* gene expression was also up-regulated during fibrosis (Wang *et al*., 2011). Overexpression of TIMPs, leading to an imbalance between MMPs and their inhibitors results in a microenvironment unfavorable to collagenolytic activity (Wang *et al*., 2011). Epithelial basement membrane integrity plays an important role in determining normal lung re-epithelialization or fibrotic response (Wang *et al*., 2011). Indeed, contact with the interstitial components seems to be a crucial event for complete and irreversible fibroblast transdifferentiation (Yen *et al*., 2016). It was proposed that during the progression of lung fibrosis MMP-9 was linked to inflammation-induced tissue remodeling, while MMP-2 was associated with impaired tissue remodeling, leading to pathological collagen deposition and interstitial lung fibrosis (Wang *et al*., 2011). Nevertheless, MMPs-dependent functions during fibrosis are not limited to effects on ECM turnover but they are also involved in regulating the activity of a range of immunoregulatory molecules (Giannandrea & Parks, 2014). As MMP-9 is a potential activator of latent TGF-β1 and, hence, might be a pro-fibrotic mediator (Giannandrea & Parks, 2014), besides being involved in the disruption of epithelial basement membranes, it can be indirectly involved in myofibroblast formation. However, in other studies evaluating MMP-TIMP pathways in the course of endometrosis, dissimilar results were obtained. No differences in MMP-2, MMP-9, MMP-14 (an enzyme capable to activate MMP-2 and MMP-9) and TIMP-2 expression between endometrial categories were referred (Aresu *et al*., 2012). Also, in another study *MMP-2*, *MMP-14* and *TIMP-2* gene expression was detected at lower levels in pathologic endometrium, whereas transcription of *MMP-9* was much higher when compared to healthy tissue values (Falomo *et al*., 2015).

7.3. Reproductive implications of endometrosis

Endometrosis is a multi-factorial disease. Age, repeated pregnancies, parturition, chronic inflammation, and endocrine problems are all factors which seem to play a determinant role in

the onset and severity of endometrosis (Hoffmann *et al*., 2009a. The occurrence of aberrations in endometrial glands, blood and lymphatic vessels; the altered endocrine milieu; and the disturbances in uterine secretory functions in endometrosis create a poor uterine environment that may contribute to embryonic loss.

Fibrosis involving the base of the glands (resulting in the formation of fibrotic nests), or surrounding the ducts of the glands, reduces the secretion flow and may result in glandular dilation and cysts formation (Walter *et al*., 2001). Reduction in the number and area of intact and healthy glands, decreases the chance of keeping a viable conceptus in the uterus (Kenney, 1978), since the exchange of nutrients and metabolic end-products via the placenta cannot be adequately ensured.

Some embryonic loss in mares suffering from endometrosis can be explained by insufficient uterine secretory activity (Hoffmann *et al*., 2009b). Collagen deposition around endometrial glands, with irregular differentiation of epithelial cell in the fibrotic glands, or associated with the basal membrane, compromises the integrity and function of those glands and impairs glandular secretions (Walter *et al*., 2001; Hoffmann *et al*., 2009b). Altered glandular secretion of histotroph, may contribute to pregnancy failure through delayed placental development, retarded fetal growth rate or abortion (Kenney, 1978; Walter *et al*., 2001; Hoffmann *et al*., 2009b; Lehmann *et al*., 2011). Also, reduced uterine blood flow and impaired fetal-maternal interactions in endometrosis-affected mares could be unfavorable for embryonic/fetal development (Klewitz *et al*., 2015). Lymphangiectasia secondary to vascular degeneration, damage to or lack of endometrial cilia (Ferreira-Dias *et al*., 1994, 1999), impairing the physiological lymphatic and mucociliary uterine clearance will interfere with mare's ability to become pregnant (LeBlanc & Causey, 2009; LeBlanc, 2010).

In severely and chronically inflamed endometria, there can be loss of the epithelium, disruption of luminal architecture, loss of uterine mucus blanket and increased opportunities for bacterial adhesion. These changes may further contribute to persistence of uterine infection (LeBlanc & Causey, 2009; LeBlanc, 2010).

In a previous work, fibrosis in the allantochorion was the main histological abnormality seen in the allantochorion-endometrium adhesion that occurred in 88% of the mares with retained fetal membranes (Rapacz *et al*., 2012). Adhesion of the allantochorion to the endometrium was correlated to abortion and preterm delivery, due to abnormalities in the process of placental tissues maturation (Rapacz *et al*., 2012). Although, in this study the presence of fibrotic lesions in the endometrium of these mares was not evaluated, putative development of allantochorion fibrosis in mares with endometrial fibrosis may also be related to abortion and preterm delivery.

7.4. Endometrosis treatment

Stem cells are characterized by their ability to self-renewal and differentiate into a diverse range of specialized cell types (Gulati *et al*., 2014). Mesenchymal stem cells (MSCs) are a heterogeneous subset of stromal cells that have the ability of self-renewal and multipotency, which could differentiate into cells of the different lineages and that have several anti-inflammatory actions (Gulati *et al*., 2014). By producing bioactive mediators and adhesion molecules MSCs reduce scar tissue formation and cell apoptosis, increase angiogenesis and stimulate the intrinsic cell population to regenerate function (Gulati *et al*., 2014). Somatic stem cells subpopulation - Side Population cells are cells that present characteristics of stem cells (Cervelló *et al*., 2010). Their presence in the mare endometrium has been shown (Pereira *et al*., 2014; Dias, 2014), although no study has determined their presence in the endometrium of mares with fibrosis.

Currently no effective treatment is available for endometrosis. Recent studies have focused on the use of MSCs to try to reverse or delay the progression of mare endometrial fibrosis. However, the results obtained in this small number of studies carried out so far are inconsistent. Mambelli and co-authors (2013) using a technique similar to artificial insemination, were able to show homing of fluorescently labeled equine adipose tissue-derived MSCs in glandular and periglandular spaces of endometrial biopsies collected 7 and 21 days after delivery of MSCs into the uterus of mares with endometrosis. Nevertheless, in one mare, with a very severe degree of endometrosis, MSCs cells could not be identified. A severely affected uterine environment preventing the incorporation of these MSCs or the damaged microenvironment might not have allowed reproducible propagation of MSCs (Mambelli *et al*., 2013). In another study, the same group showed that equine adipose tissue-derived multipotent mesenchymal stem cells (eAT-MSCs) were able to positively modulate the expression pattern of secretory proteins. In addition, they were also able to induce glandular epithelial cells proliferation suggesting local benefits to committed endometrial tissue environment after eAT-MSCs transplantation (Mambelli *et al*., 2014). Expression of α-SMA cells were no longer observed in endometrial uterine glands of fibrotic tissues, 7 days after the animals received eAT-MSCs treatment, inducing an early remodeling of endometrial tissue microenvironment (Mambelli *et al*., 2014). It was suggested that the preventive use of stem cells therapy by slowing down the degenerative process may be used in those mares with severe endometrial degeneration that had not responded to treatment (Mambelli *et al*., 2014).

However, in another study it was not possible to detect a clear effect of autologous bone marrow (BM)-MSCs transplanted into the endometrium of mares with endometrosis in the

expression of the profibrotic cytokine TGF-β1 and its receptors TGFBRI and TGFBRII (Cardoso, 2015; Cardoso *et al*., 2016). After infusion of autologous BM-MSCs in the endometrium of 7 mares with endometrosis, a decreased intensity of TGF-β1 and TGFBRI at D30 and D60 was only detected in 2 mares but the expression of TGFBRII did not change after BM-MSCs treatment (Cardoso, 2015; Cardoso *et al*., 2016). Likewise, no clear potential clinical effect of equine adipose-derived stem cells (ADSCs) on equine endometrosis in *in vitro* experiments were noted in Falomo *et al*., (2015) study. Although ADSCs were able to be incorporated into endometrial periglandular space and in single glands, both positive and negative effects of equine ADSCs on inflammatory processes regulation in the endometrium was noted (Falomo *et al*., 2015). Fibrotic endometrial tissues exposure to the ADSCs resulted in a reduction of IL1-β, TNFα, IL-10 and IL1RN, downregulating chronic inflammation, but it also increased the levels of pro-inflammatory IL-6 and IL-8 (Falomo *et al*., 2015).

In view of these results, it seems unlikely that local infusion of MSCs will reverse severe fibrotic changes. However, there may be beneficial effects in its use, delaying the evolution of the fibrotic process in endometrial tissues with less severe degenerative changes. Further longer studies are needed with a larger number of animals, using different MSC protocols (source and amount of MSC, phase of the estrous cycle in which local MSC treatment is applied, number of required infusions, simultaneous MSC treatment by systemic route, among others). Besides, further investigation on the effects of MSC on additional inflammatory/fibrotic markers in order to draw conclusions regarding the putative efficacy of MSC use as a means to delay or prevent the development of endometrosis.

Chapter III- *Experimental Work*

1. Endometrial prostaglandin synthases, ovarian steroids and oxytocin receptors in mares with oxytocin induced luteal maintenance

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1.1. Abstract

Oxytocin (OXT) has been used to prolong the luteal phase in mares, but its mechanism of action is unknown. The aim of this study was to evaluate the effect of chronic exogenous oxytocin administration to mid-luteal phase mares on luteal maintenance. Also, endometrial expression of prostaglandin endoperoxide synthase 2 (PTGS2), prostaglandin $F_2\alpha$, E_2 and I_2 synthases (AKR1C3, PTGES, PTGIS), receptors for oxytocin (OXTR), progesterone (PGR), estrogen receptors 1 (ESR1) and 2 (ESR2) were assessed in mares experiencing luteal maintenance two weeks after chronic exogenous OXT administration. Control mares (n=5; C-group) received 6 mL of saline im, while OXT (60 units/mare) was administered im (n=6; OXT-group), every 12h, on days 7-14 post-ovulation. After endometrial biopsy in groups C (day 10) and OXT (day 24), luteolysis occurred within 3 or 6 days, respectively. Luteal maintenance took place in 4/6 (67%) of OXT treated mares. Progesterone in C-group was the highest on biopsy day ($P < 0.05$). In OXT mares, *PTGS2, ESR1* ($p<0.05$), *PTGES, PTGIS, PGR, ESR2* ($P < 0.01$) gene transcription decreased, while *OXTR* increased ($P < 0.05$) in comparison to C-group. In OXT treated mares, endometrial ESR2 protein expression decreased ($P < 0.05$), but OXTR increased ($P < 0.05$), when compared to control animals. In both experimental groups, PTGS2 was mainly immunolocalized in surface epithelium, while AKR1C3, PTGES, PTGIS and PGR were in surface and glandular epithelia. ESR1 and ESR2 were found in glandular epithelium, and OXTR in stromal cells. High immunolabeling for PTGES, PTGIS, PGR and OXTR and low for ESR2 was detected in endometrium of OXT-group mares with extended diestrus. Prolonged luteal function associated with chronic OXT treatment may be related to different spatial expression of OXTR and PGR in the endometrium. The observed reduction of endometrial ESR2 may be responsible for the maintenance of PGR in luminal and glandular epithelium. Also, ESR2 may attenuate the transcriptional activity of *ESR1* in mare endometrium. This study offers new knowledge on the endometrial expression of ovarian steroids and OXT receptors in OXT pharmacologically induced luteal maintenance in the mare.

Keywords: mare, luteal phase, oxytocin, endometrium, steroids receptors**,** oxytocin receptor.

1.2. Introduction

Oxytocin (OXT) exerts a variety of actions and is involved in a large number of physiologic processes, such as regulation of luteal function. Oxytocin is stored in posterior pituitary vesicles and released into the peripheral circulation in high frequency bursts in late diestrus (Vanderwall *et al*., 1998). In the mare, this hormone is also synthesized by endometrial cells and secreted into the uterine lumen where it has an important role in the autocrine/paracrine control of uterine contractility and luteolysis (Watson *et al*., 2000). Endometrial OXT concentrations are positively correlated with serum estradiol-17β (E2) concentration (Behrendt-Adam *et al*., 1999, Bae & Watson, 2003). Nevertheless, hormonal regulation of OXT receptors (OXTR) in equine endometrium appears to differ from ruminants (Fleming *et al*., 2006), since in mares OXTR expression is low at estrus (Sharp *et al*., 1997; Ruijter-Villani *et al*., 2015).

Luteolysis occurs on days 14 to 16 of the equine estrous cycle (Ginther *et al*., 2011). Although E2 is apparently not required for luteolysis in mares (Ginther *et al*., 2005), in other mammals, E2 is considered a key regulator of *OXTR* gene expression (Kimura *et al*., 2003). In the mare, E2 plasma concentrations begin to increase approximately 2 days before the onset of luteolysis (Ginther *et al*., 2005). In late luteal phase (day 14-15), *OXT* gene expression (Behrendt-Adam *et al*., 1999) and OXTR concentration in mare endometrium increase and mediate prostaglandin (PG) release (Sharp *et al*., 1997; Starbuck *et al*., 1998). Afterwards, OXTR activation stimulates mitogen activated protein kinase (MAPK), which regulates prostaglandin-endoperoxide synthase gene expression (Soloff *et al*., 2000). At day 15, an increase in both mRNA and protein levels of PTGS2 in luminal epithelium has been detected (Boerboom *et al*., 2004; Atli *et al*., 2010). These enzymes convert arachidonic acid to PGH2, which can be converted to PGF2α by AKR1C3 in the uterus. OXT binding to its receptor is responsible for stimulating both frequency and amplitude of endometrial surges of PGF2α necessary for luteolysis (Sharp *et al*., 1997; Starbuck *et al*., 1998). In response to PGF2α luteolytic pulses over a 23-h period, the CL undergoes functional and structural regression (luteolysis), and progesterone (P4) progressively decreases to values below 1ng/mL (Ginther *et al*., 2011).

Although endogenous OXT action is involved in PGF2α endometrial secretion during spontaneous luteolysis in mares (Sharp *et al*., 1997; Starbuck *et al*., 1998; Vanderwall *et al*., 2007), PGF2α response to exogenous OXT treatment depends on the phase of the estrous cycle (Neely *et al*., 1979; Vanderwall *et al*., 2016). Although PGF2α endometrial secretion is highest around the time of luteolysis (days 11-15 post-ovulation) and is associated with an increase in endometrial OXTR expression (Sharp *et al*., 1997; Starbuck *et al*., 1998), when

exogenous OXT is administered in mid luteal phase (day 8 to 14), it blocks luteolysis and prolongs the luteal phase (Stout *et al*., 1999; Vanderwall *et al*., 2007; Vanderwall *et al*., 2012; Gee *et al*., 2012; Keith *et al*., 2013). The exact mechanism involved in this action is unknown. Since ovarian steroid hormones are essential mediators of utero-ovarian function acting through specific receptors and PG pathways, their relationship with OXT throughout the mare estrous cycle has to be considered. As in other mammalian species, in mare endometrium, E2 produced in the follicular phase up-regulates its own nuclear receptors (ESR), as well as P4 receptors (PGR) (Hartt *et al*., 2005). The effects of E2 are exerted in the endometrium via ESR1 and ESR2, which are the two-main classical nuclear receptor isoforms (Hapangama *et al*., 2015). Although there are two predominant isoforms of nuclear PGR (PGR-A and PGR-B), PGR-A is the major functional isoform in the uterus (Conneely & Lydon, 2000). In the mare, during estrus, high endometrial levels of ESR1, ESR2 and PGR mRNA and protein have been detected in luminal, glandular epithelia and stromal cells (Watson *et al*., 1992; Hartt *et al*., 2005; Honnens *et al*., 2011; Gebhardt *et al*., 2012; Silva *et al*., 2014). Moreover, the relative mRNA expression of *ESR1* and *ESR2* in mare endometrium seems to be positively correlated (Honnens *et al*., 2011). In the luteal phase, when circulating levels of P4 are high, endometrial expression of ESR1, ESR2 and PGR are inhibited (Hartt *et al*., 2005). This expression decreases in stroma and deeper glandular epithelium and does not exist in luminal epithelium on days 11 and 14 of the mare estrous cycle (Hartt *et al*., 2005). Between days 17 to 20 of the estrous cycle, both ESR and PGR transcripts and protein increase in luminal and glandular epithelia and in stromal cells (Hartt *et al*., 2005). We hypothesized that chronic OXT administration to mares in mid-luteal phase would be able to prolong luteal function by modulating PG pathways, steroid hormones and OXT receptors in the endometrium. Several studies have implicated those endometrial pathways in the beginning of OXT induced luteal maintenance (Day 14 post-ovulation) (Stout *et al*., 1999; Vanderwall *et al*., 2007; Vanderwall *et al*., 2012; Keith *et al*., 2013). Thus, the aim of the present study was to evaluate the endometrial expression of PTGS2, AKR1C3, PTGES, PTGIS, OXTR, PGR, ESR1 and ESR2 following the administration of exogenous OXT in mid to late luteal phase (day 7 to 14 after ovulation) to induce luteal maintenance.

1.3. Materials and methods

1.3.1. Animals

This study was carried out on Lusitano mares aged from 3 to 17 years, weighing between 450 and 500 Kg. All had normal estrous cycles throughout the study period (between early June and early August). The animals were housed in boxes, fed grass hay and commercial

concentrate mix with free access to trace mineral salt blocks and water. All mares were routinely subjected to regular husbandry procedures, such as deworming and vaccinations. The protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal.

1.3.2. Experimental design

Throughout the entire experimental period, all mares were teased daily by a single stallion to detect estrous behavior. Each mare's genital tract was evaluated every-other-day by transrectal palpation and ultrasonography (Falco, 6MHz linear transducer) for detection of ovulation (day 0), *corpus luteum* (CL) size monitoring and signs of estrus return (endometrial edema). A crossover design was used, so that the same animal received each of the two treatments sequentially. On day 7 of the estrous cycle, mares were randomly assigned to two groups: control group (n=5; C-group) and oxytocin group (n=6; OXT-group). On days 7-14 after ovulation, OXT mares received 60 units (10 units/mL) of oxytocin, im, every 12 hours; control mares received 6 mL of saline solution im on the same schedule. Blood samples were obtained every-other-day via jugular venipuncture.

1.3.3. Endometrial biopsies

Nonsurgical endometrial biopsies were collected using sterile uterine alligator jaw forceps guided through the cervix into the uterus. Two biopsies per mare were obtained from the ventral wall at the base of each uterine horn. We have observed that endometrial biopsy in mares in mid-luteal phase stimulates luteolysis in about 3 days (unpublished data), thus obtaining endometrial samples for evaluation of causes for OXT-stimulated luteal maintenance disturbed the process of luteal maintenance. Collecting endometrium at the time of expected endometrial secretion of PGF2 in response to OXT (endogenous or exogenous) (Sharp *et al*., 1997; Starbuck *et al*., 1998) in control mares to compare with samples obtained following confirmation of OXT-stimulated luteal maintenance was therefore necessary. Control group endometrial biopsies were performed on day 10 post-ovulation, when a functional and structurally mature CL was present (Ferreira-Dias *et al*., 2006). In OXT treated mares, biopsies were obtained following luteal maintenance for 8-10 days beyond the expected time of normal luteolysis, on day 24, which was the earliest feasible time to confirm a retained CL; spontaneously prolonged luteal function in mares persists for at least 30 and often 60 days post-ovulation (Vanderwall *et al*., 2007; Vanderwall *et al*., 2012). Thus, comparisons were made of endometrial conditions before luteolysis in saline treated mares and after initiation of prolonged luteal function in OXT treated mares.

Biopsy samples were divided and placed in RNA stabilization reagent (RNAlater®, 76104, Qiagen, Hilden, Germany) for mRNA assessment (Real Time PCR); stored at –80º C for protein quantification (Western blot); or placed in 4% buffered formaldehyde for histologic evaluation and immunohistochemistry. Samples preparation included fixing with formaldehyde overnight, dehydration in a series of ethanol dilutions, xylene infiltration, and embedding in paraffin. Tissue was cut into 4 μm histological sections and stained with hematoxylin eosin for tissue microscopic evaluation.

1.3.4. Analysis of blood samples

Blood samples were collected using heparinized tubes (monovettes®, Sarstedt, Numbrectht, Germany). Plasma obtained after centrifugation was kept frozen at -20ºC until further analysis. Circulating concentrations of P4 were assayed in duplicate and measured by a validated solid-phase Radioimmunoassay (RIA), without extraction, using a commercial kit (Coat-A-Count; Diagnostic Product Corporation, Los Angeles, CA, USA), and a Wallac (Wizard 1470) counter. The limit of detection of the assay was 0.02 ng/mL, all samples were run in the same assay and the intra-assay coefficient of variation for all samples was 3.4%. Circulating P4 concentrations were used to evaluate luteal function. Concentrations higher than 1 ng/mL were considered indicative of functional luteal tissue.

1.3.5. Real-Time PCR

Real Time PCR was used to assess mRNA gene expression of *PTGS2, AKR1C3, PTGES, PGIS, OXTR, ESR1, ESR2* and *PGR*, as described (Galvão *et al*., 2012). Briefly, RNA extraction from frozen endometrial biopsy samples was performed using a Total RNA Extraction and Purification kit (ref. 74124, Qiagen GmbH, Germany), and DNA digestion with RNase-free DNase Set (ref. 50979254, Qiagen, Germany). Reverse transcription by superscript III enzyme (N2511, Promega, Madinson, USA), was carried out from 0.8 μg total RNA in a 20 μL reaction volume, using oligo (dT) primer (C1101, Promega, Madinson, USA) and cDNA was stored at -20ºC. Specific primers for *PTGS2, AKR1C3, PTGES, PTGIS, OXTR, ESR1, ESR2, PGR* and reference genes were designed (Table 1), as before (Galvão *et al*., 2010). Four potential reference genes were tested to determine the most stable internal control gene for our endometrial samples: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), succinate dehydrogenase A complex, subunit A, flavoprotein (*SDHA*), beta-2-microglobulin (*B2M*) and ribosomal protein L32 (*RPL32*). *RPL32* was found to be the most stable internal control gene (less than 2-fold changes between different biological conditions) (Dheda *et al*., 2004). After primer concentration optimization, Real-time PCR assays were

performed using the 7300 Real Time PCR System. Detection of PCR products was achieved with DNA-intercalating dye SYBR Green I, in a 25uL reaction volume. Relative mRNA quantification data were analyzed with the Real-time PCR Miner algorithm (Zhao & Fernald, 2005). Expression of target genes was normalized against that of reference gene and mRNA expression compared between control and OXT treated mares.

Table 1- Primer sequences used for prostaglandin synthases, ovarian steroids and oxytocin receptors real time PCR analysis of mare endometrial biopsies.

Gene (Acession number)	Sequence 5´- 3	Amplicon (base pairs)
PTGS2 (GeneID: 791253)	Forward: TGCTGTTCCAACCCGTGTC	204
	Reverse: GACAATGTTCCAGACTCCCTTGA	
AKR1C3 XM_001500921.1	Forward: TGGGTCACTTTCCTTCAACCA	200
	Reverse: CTTCTCCATTGCCTCCCATGT	
PTGES (NM_001081935.1)	Forward: CACGCTGCTGGTCATCAAGA	127
	Reverse: GGTCGTCCCGGTGAAACTG	
PTGIS XM_001501166.2|	Forward: ACCACCCTCCCACAGAAGGT	177
	Reverse: AAAGAGGAGGAGGCGGTCAC	
OXTR (XM_001491665.2)	Forward: TGGACGCCATTCTTCTTCGT	141
	Reverse: GCCCGTGAACAGCATGTAGA	
ESR1 (GeneID: 791249)	Forward: ACGATGCCACCAGACCATTT	160
	Reverse: AGCCAGGCACATTCCAGAAG	
ESR2 (GeneID: 100033964)	Forward: CCCTTCACCGAGTCCTCCAT	232
	Reverse: TCCCTGTCCAGAACGAGGTC	
PGR (GeneID: 100033883)	Forward: CCCAGCATGTCGCCTTAGAA	150
	Reverse: AGGGGTTGGCTTTCATTTGG	
RPL32 (XM_001492042.6)	Forward: AGCCATCTACTCGGCGTCA	144
	Reverse: GTCAATGCCTCTGGGTTTCC	

AKR1C3- aldo-keto reductase family 1, member C3; *ESR1*- estrogen receptor 1; *ESR2*- estrogen receptor 2; *OXTR*- oxytocin receptor; *PGR*- progesterone receptor; *PTGES*- microsomal prostaglandin E2 synthase 1; *PTGIS*- prostaglandin I2 synthase; *PTGS2*- prostaglandin-endoperoxide synthase; *RPL32*- ribosomal protein L32;

1.3.6. Immunohistochemistry analysis

The presence of PTGS2, AKR1C3, PTGES, PTGIS, OXTR, ESR1, ESR2 and PGR was assessed by immunohistochemistry (IHC). The detection was performed on 4 µm endometrial sections by an immunoperoxidase method using specific antibodies and NovoLink Max Polymer Detection System Kit (Leica Biosystems Newcastle Ltd, Newcastle, United Kingdom), as previously described (Galvão *et al*., 2013). Primary antibodies dilution, incubation time and temperature are specified on Table 2. The primary antibody was detected by incubating samples with anti-mouse/rabbit IgG -Poly-HRP (each at 8µg/mL) containing 10% (v/v) animal serum in TBS/0.09%ProClinTM 950 for an additional 30 min. Staining was achieved by a 5min incubation in 3,3-diaminobenzidine (DAB) peroxidase substrate solution. Slides were counterstained with hematoxylin prior to mounting.

Table 2- Primary antibodies dilution, incubation time and temperature used in immunohistochemistry and western blotting analysis of mare endometrial biopsies.

	Immunohistochemistry		Western blot	
Primary antibody	Dilution	Incubation Time/Temperature	Dilution	Incubation Time/Temperature
PTGS2	1:100	ON, 4ºC	1:250	ON, 4ºC
AKR1C3	1:100	ON, 4ºC		
PTGES	1:100	ON, 4ºC		
PTGIS	1:100	ON, 4ºC		
OXTR	1:500	1h, RT	1:1000	1 h, RT + 0,5h, 37ºC
ESR1	1:100	ON, 4ºC	1:500	ON, 4ºC
ESR2	1:500	ON, 4ºC	1:1000	ON, 4ºC
PGR	0,6µg/ml	ON, 4ºC		

PTGS2- mouse anti-rat COX2 antibody, Clone 33, (610204, BD Biosciences); PGFS rabbit Anti-AKR1C3 antibody (ab137546, abcam); PTGES- rabbit Prostaglandin E Synthase-1 (microsomal) Polyclonal Antibody (160140, Cayman Chemical); PGIS- rabbit Prostaglandin I Synthase Polyclonal Antibody (100023, Cayman Chemical); OXTR- rabbit Oxytocin-R (H60) Antibody (SC 33209, Santa Cruz biotechnology); ERα- rabbit anti- Estrogen Related Receptor alpha antibody (ab 16363, abcam); ERβ- rabbit anti-Estrogen Receptor beta antibody (ab 3577, abcam); PR- mouse anti-Progesterone Receptor, Clone 1A6 (PR08-1150, Invitrogen); ON- overnight; RT-room temperature.

Negative controls were performed with primary antibody replaced by rabbit polyclonal IgG (ab 27478, Abcam) and mouse IgG (550878, BD Bioscience) for antibodies developed in rabbit and mouse respectively, at the same concentration and incubation time. Immunostaining appeared as a characteristic brown staining, and was assessed by light microscopy (Olympus BX51, Tokyo, Japan) equipped with a DP11 Olympus camera (Tokyo, Japan). Luminal and glandular epithelial and stromal staining area and intensity were assessed on 10 random fields by three evaluators blinded to treatment groups. Intensity of immunolabeling was quantified by a subjective score (1. no staining; 2. weak staining; 3. moderate staining; 4. intense staining). Scores from all evaluators were averaged for each endometrium slide.

1.3.7. Western blot analysis

Western blot was used to quantify PTGS2, ESR1, ESR2 and OXTR protein expression. Protein extracts were prepared from frozen endometrial biopsy samples. Briefly, tissues were homogenized on ice in RIPA buffer (50 mM Tris-HCl, pH 8; 150 mM NaCl; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulphate) with protease inhibitors (Complete Mini Protease Inhibitor Cocktail Tablets, 1 tablet per 10 ml of buffer; Roche) and disrupted using an Ultrasonic homogenizer (Bandelin Sonopuls; Berlin, Germany). Protein content of the supernatants was quantified using the Bradford (ref. 500-0006, Bio-Rad, Hercules, CA, USA) method, and 100 µg of protein was separated by SDS-PAGE (10%

acrylamide gel; ref. 161-0155, Bio-Rad, Hercules, CA, USA). Gels were subsequently transferred to nitrocellulose membranes (Ref. 10401396, Whatman; GE Healthcare Europe GmbH, Lisbon, Portugal) and incubated with the same specific primary antibodies used for immunohistochemistry analysis. Table 2 lists the specific dilution, temperature and time of incubation of primary antibody. To normalize loaded protein, diluted (1:10,000) mouse monoclonal antibody against β actin (A5441, Sigma) was used. Incubation with secondary antibodies was performed, for 2.5h at room temperature, with 1:20,000 diluted horseradish peroxidase (HRP)-conjugated anti-rabbit (P044801- Dako, Denmark) for all rabbit produced primary antibodies and HRP-conjugated goat anti-mouse (A2554, Sigma) for PTGS2, PGR and for β-actin (1:10,000 dilution). Chemiluminescent detection was achieved by incubation of the membrane with SuperSignal® West Pico (34077, Thermo Scientific, Waltham, MA, USA) and its exposure to a photographic film (Kodak BioMax Light Film; Kodak-Industrie, Chalon-sur-Saone, France). Target proteins expression was normalized dividing the units of arbitrary densitometry by β-actin density for each band. ImageJ (http://rsb.info.nih.gov/ij/index.html) was used to evaluate densitometry signals (Miller, 2010).

1.3.8. Statistical analyses

Two-tailed unpaired t-test was used to analyze plasma P4 concentration, day of luteolysis after biopsy, gene and western-blot and immunohistochemistry protein expression data. In order to detect differences between groups in plasma P4 levels in the first 11 days after ovulation, a repeated measures ANOVA followed by Bonferroni comparison test was used. Mean differences were calculated with 95% confidence interval and differences were considered significant when $P < 0.05$. Statistical analyses were performed using GraphPAD PRISM (Version 5.00, GraphPad Software, San Diego, CA, USA). Results were expressed as means ±SEM.

1.4. Results

1.4.1. Estrous cycle length and plasma progesterone concentration

One mare in control group underwent luteolysis during the treatment period and was therefore excluded from the study. Two of the mares subjected to exogenous OXT administration experienced luteolysis (OXTRE-group) in the last day of treatment (day 14) and were also excluded from further gene and protein endometrial studies. Prolonged luteal function occurred in 4 of the 6 (67%) mares in OXT-group, as demonstrated by CL persistence in the ovaries, absence of endometrial edema (Fig. 2A, B) and plasma P4 concentrations >1ng/ml

(Fig. 2C). Plasma P4 concentrations between control and OXT-group did not differ ($P = 0.42$) in the first 11 days after ovulation. Plasma P4 was higher (13.7±1.5 ng/mL) in C-group, than in the OXT-group (8.33±1.8 ng/mL; $P < 0.05$) on biopsy day ($P< 0.05$). In mares with OXT-induced prolonged luteal function (n=4), plasma P4 levels remained higher than 1ng/mL throughout 30 days post-ovulation. Luteolysis in response to biopsy was significantly delayed in the OXT treatment group compared with controls (3.2±0.2 for Controls and 6.0±1.4 days for OXT group; $P < 0.05$).

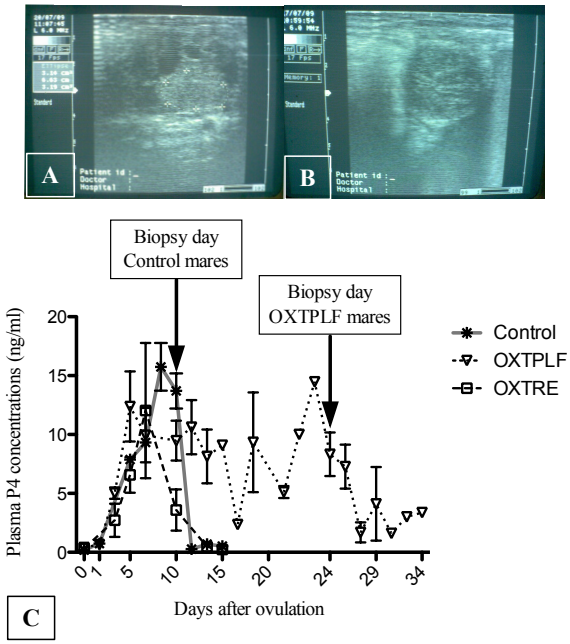

Figure 2- *Corpus luteum* (**A**) and uterus (**B**) 24 days post-ovulation and plasma progesterone concentration (**C**) in oxytocin treated mares with prolonged luteal function. Values represent mean ± SEM. Control- mares treated with saline; (n=4); OXT- mares treated with 60 units of oxytocin/mare (n=6); OXTPLF- oxytocin treated mares with prolonged luteal function (n=4); OXTRE- oxytocin treated mares that underwent luteolysis at the normal time (n=2); P4- progesterone.

All mares with OXT-extended luteal function presented a functional CL (confirmed by transrectal ultrasound and by plasma P4, plus an absence of ovarian follicles with meaningful sizes (< 25 mm), similar to mid-diestrus. Endometrial histology revealed characteristic features of diestrus (higher density and tortuosity of glands with a "string of pearls" appearance (Kenney, 1978).

1.4.2. Prostaglandin synthases, ovarian steroids and oxytocin receptors mRNA transcription

When comparing mRNA transcription of enzymes involved in endometrial prostaglandin biosynthesis, a reduction in *PTGS2* ($P < 0.05$), *PTGES* and *PTGIS* ($P < 0.01$) was observed in OXT treated mares, while *AKR1C3* was similar between groups (Fig. 3). Transcription levels of *ESR1* ($P < 0.05$), *ESR2* and *PGR* ($P < 0.01$) genes decreased, whereas *OXTR* increased ($P < 0.05$) in OXT-treated mares, when compared with control group (Fig. 3).

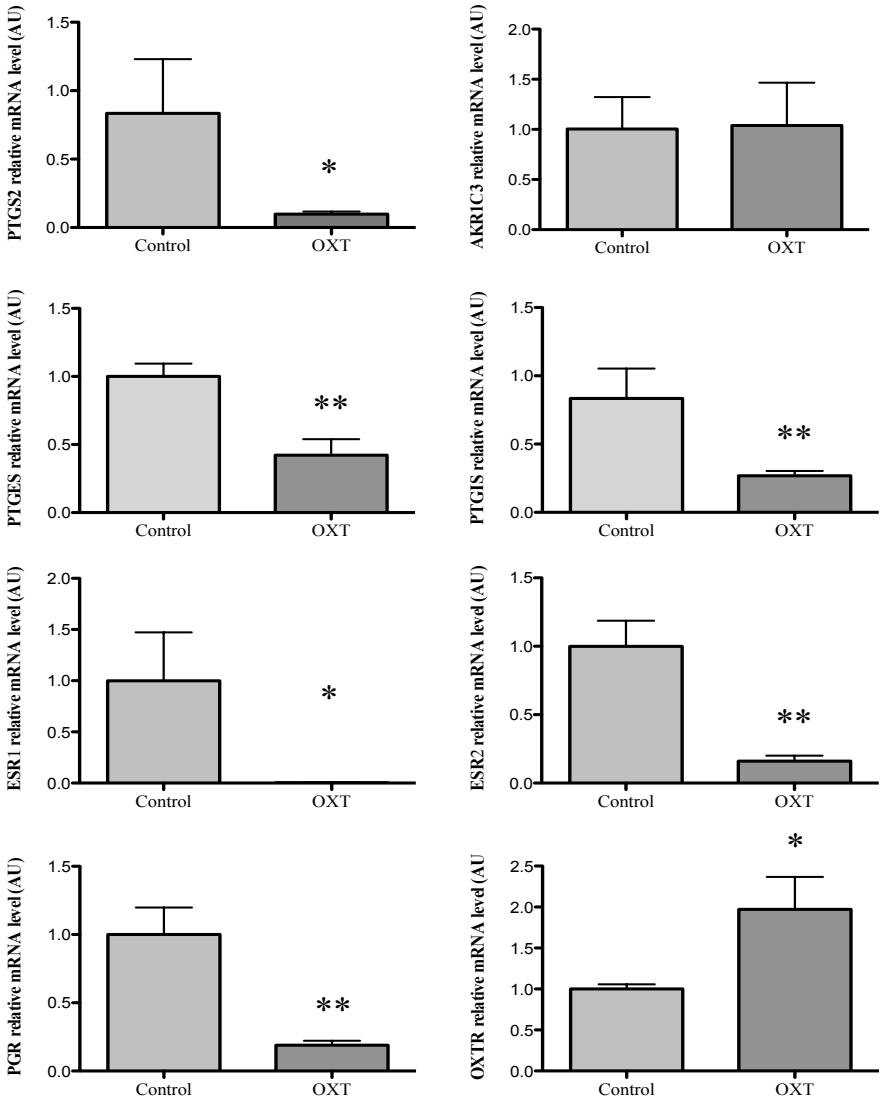

Figure 3- Relative quantification of gene transcription by real-time PCR of proteins involved in prostaglandin biosynthesis and oxytocin and ovarian steroids receptors in endometrial biopsies of control and oxytocin treated mares. Endometrial biopsies were collected on day 10 post-ovulation in control mares (n=4) and on day 24 in oxytocin treated mares (n=4). Expression of target genes was normalized against that of the housekeeping gene (RPL32). Bars represent mean ± SEM. AU: arbitrary units. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$). *AKR1C3*- aldo-keto reductase family 1, member C3*; ESR1*- estrogen receptor 1*; ESR2*- estrogen receptor 2*; OXTR*- oxytocin receptor; *PGR*- progesterone receptor; *PTGES*- microsomal prostaglandin E synthase 1*; PTGIS*- prostaglandin I2 synthase*; PTGS2*- prostaglandin-endoperoxide synthase.

1.4.3. Prostaglandin synthases, oxytocin and ovarian steroids receptors protein expression and immunolocalization

Western blot and IHC analyses were performed to evaluate differences in biosynthesis of endometrial PG enzymes and receptors for OXT and ovarian steroids between control and OXT-treated prolonged CL mares.

Protein expression analysis by western blot was performed for PTGS2, ESR1, ESR2 and OXT receptors. No significant differences were detected in band intensity of PTGS2 (70 kDa molecular weight–MW band) and ESR1 (52 kDa MW band) between control and OXT treated mares. However, there was a decrease in expression level of ESR2 ($P < 0.05$; 59 kDa MW band) and an increase of OXTR ($P < 0.05$; 66 kDa MW band) in OXT-treated mares compared to control mares (Fig. 4).

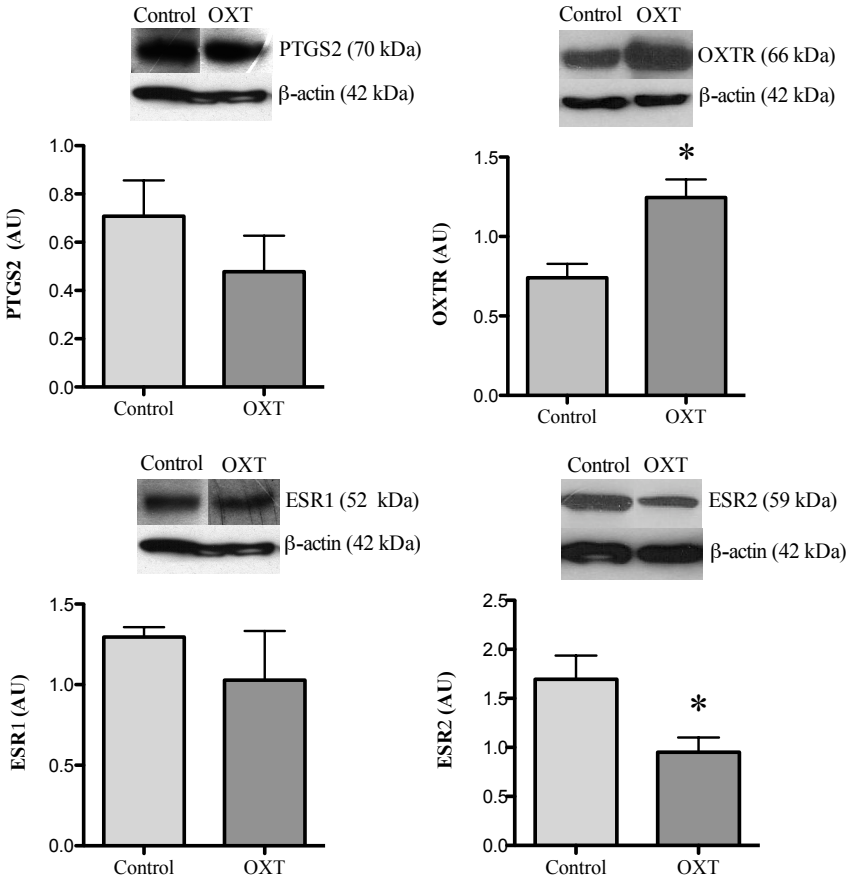

Figure 4- Protein expression of PTGS2, oxytocin receptor (OXTR), and estrogen receptors (ESR1, ESR2) in endometrial biopsies of control and oxytocin treated mares. Endometrial biopsies were collected on day 10 post-ovulation in control mares (n=4) and on day 24 in oxytocin treated mares (n=4). Histograms show densitometric analysis of PTGS2, OXTR, ESR1 and ESR2 proteins normalized against β-actin. Upper panels illustrate representative western blots. The left lane of each blot is from control mares and the right from OXT treated mares. Data represent mean ± SEM. AU: arbitrary units. Asterisk indicates significant differences (* $P < 0.05$).

Results of IHC are shown in Fig. 5 and 6. PTGS2 was detected in surface epithelium and stromal cells, with occasional labeling of glandular epithelium. Immunostaining was the strongest in luminal surface epithelium for all mares (Fig. 5). Immunolabeling for AKR1C3, PTGES and PTGIS was found in superficial and glandular epithelia in both control and OXT treated mares. While no differences were found in the intensity of immunostaining for AKR1C3 between groups, OXT-treated mares showed a tendency for a larger stained glandular area compared with the C-group (P = 0.06; Fig. 5). High immunolabeling of PTGES and PTGIS was detected in OXT-treated mares ($P < 0.05$; Fig. 5).

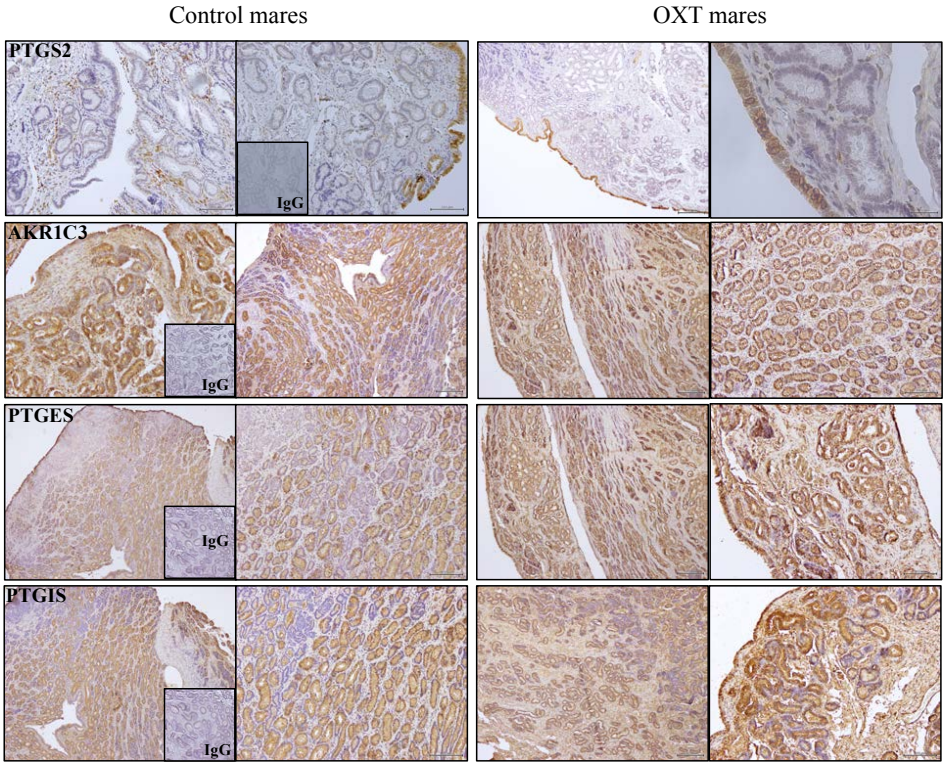

Figure 5- Endometrial immunohistochemistry representative images of PTGS2, AKR1C3, PTGES and PTGIS protein in endometrial biopsies of control and oxytocin treated mares. Endometrial biopsies were collected on day 10 post-ovulation in control mares and on day 24 in oxytocin treated mares (OXT). Positive staining is shown in brown. In negative controls, primary antibodies were replaced by the same concentration of rabbit or mouse IgG.

Progesterone receptor was present in luminal and glandular epithelium, with more intense immunostaining in the OXT-treated group compared to the control ($P < 0.05$; Fig. 6). Immunostaining for OXTR was the strongest in stromal cells with scarce staining present in superficial glandular epithelium. Prolonged CL mares treated with OXT had increased OXTR expression compared with control mares ($P < 0.05$; Fig. 5). Immunolabeling for ESR1 and ESR2 was found in glandular epithelium in both groups. While no differences were observed in ESR1 immunostaining intensity between groups, OXT treated mares showed decreased endometrial ESR2 immunoexpression ($P < 0.05$; Fig. 6).

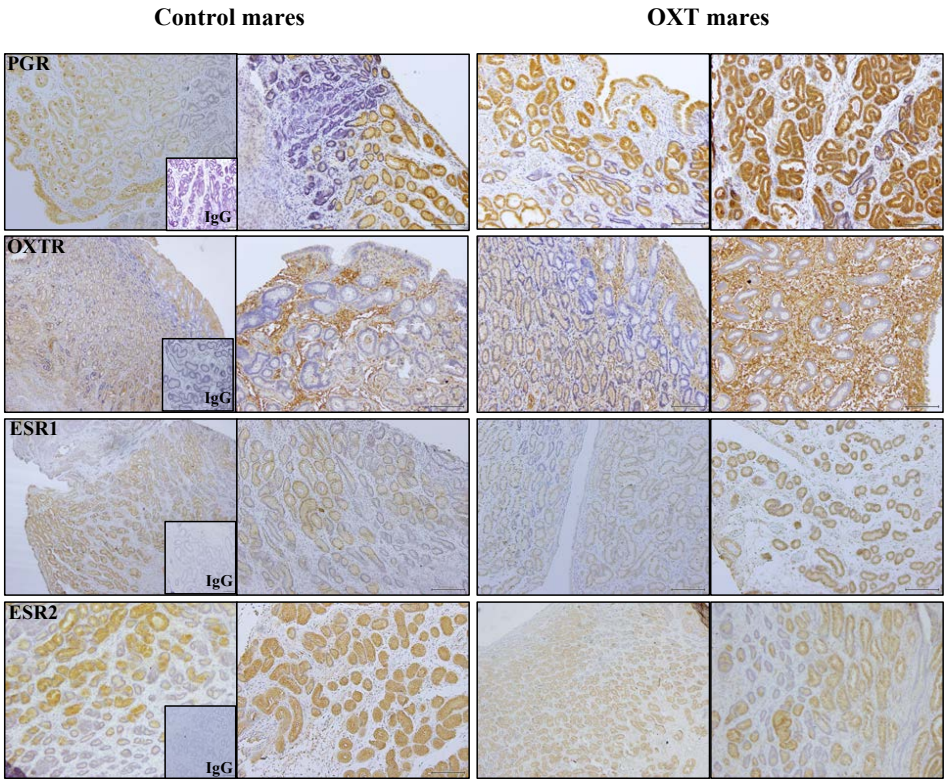

Figure 6- Endometrial immunohistochemistry representative images of PGR, OXTR, ESR1 and ESR2 protein in endometrial biopsies of control and oxytocin treated mares. Endometrial biopsies were collected on day 10 post-ovulation in control mares and on day 24 in oxytocin treated mares (OXT). Positive staining is shown in brown. In negative controls, primary antibodies were replaced by the same concentration of rabbit or mouse IgG.

1.5. Discussion

Prevention of luteolysis in mares by administration of exogenous OXT on days 8 to 14 of estrous cycle has already been described (Stout *et al*., 1999; Vanderwall *et al*., 2007; Vanderwall *et al*., 2012; Gee *et al*., 2012; Keith *et al*., 2013), however, treatment outcome seemed to depend on OXT dose and route of administration. In the present study, twice daily im administration of 60 units of OXT prolonged the luteal phase in four of six (67%) mares. This dosage and route of administration was based on other studies that reported higher success rates: 6/6 mares (100%) (Vanderwall *et al*., 2007); 5/7 mares (71.4%) (Vanderwall *et al*., 2012); or 6/7 mares (86%) (Keith *et al*., 2013). In another study, continuous administration of 27.5 units/h of OXT by sc mini-pump on days 8-20 post ovulation abolished luteolysis in 4/5 mares (80%) (Stout *et al*., 1999). However, low doses of OXT (10 units/day) on days 7-14 post-ovulation did not increase the proportion of mares with prolonged diestrus when given im, but, iv administration of the same dose induced luteostasis in 5/6 animals (86%) (Gee *et al*., 2012). The iv route allows OXT to reach endometrial tissue before being metabolized (Keith *et al*., 2013). Thus, the amount of OXT getting to endometrial cells might

be crucial to induce luteal maintenance.

A putative mechanism by which exogenous OXT might induce CL maintenance is by modifying the expression of the enzymes involved in PG synthesis. Pregnancy maintenance requires the inhibition of the luteolytic mechanism during late diestrus; this physiological state could perhaps serve as a model for the mechanism by which luteal maintenance is achieved in the absence of conception. PTGS2 expression was higher in endometrial surface epithelium during luteolysis (day 15) but not in 15-day pregnant mares (Boerboom *et al*., 2004) and *PTGS2* transcription was increased on day 15 in cyclic mares, while it was suppressed by pregnancy on days 15, 18, and 22 (Atli *et al*., 2010). Conversely, however, another study reported a marked increase in PTGS2 gene and protein expression between days 14 and 21 of pregnancy (Ruijter-Villani *et al*., 2015).

Daily oxytocin administration during the mid-luteal phase stimulates luteal retention (Stout *et al*., 1999; Vanderwall *et al*., 2007; Vanderwall *et al*., 2012; Gee *et al*., 2012; Keith *et al*., 2013). In the present study, down-regulation of *PTGS2* transcription was observed in OXT-treated mares, while PTGS2 protein in endometrial luminal epithelia did not differ between control and OXT-groups. Gene and protein expression decrease of PTGS2 in OXT-treated mares at the time that they were presumed to be in the beginning of luteal maintenance was reported (Keith *et al*., 2013). In summary, PTGS2 seems to be up-regulated in luteolysis, but down-regulated during luteal maintenance and early pregnancy (Katila, 2011). Any form of endometrial irritation during the late luteal phase, through biopsy or sterile saline infusion, results in a consequent release of PGF2α and luteolysis (Baker *et al*., 1981; King *et al*., 2010). In these aforementioned studies, successful blocking of the luteolytic process using OXT could not be confirmed due to the timing of endometrial tissue procurement and its stimulation of luteolysis as a consequence. Most prior studies report a majority, but not 100%, of mares respond to OXT treatment with a prolonged CL. Therefore, it can be assumed that the conditions assessed in the endometrial tissues collected during the late luteal phase from OXT-treated mares were not uniformly reflective of those leading to failure of luteolysis.

In the present study, we sought to compare mid-luteal phase endometrial conditions prior to luteolysis with conditions following failed luteolysis induced by OXT administration. Since OXT administration during diestrus is largely, but not always, successful in inhibiting luteolysis (Stout *et al*., 1999; Vanderwall *et al*., 2012; Gee *et al*., 2012), confirmation of the effectiveness of the treatment (prolongation of luteal function), and elimination of those mares from the analysis that did not respond was important. Due to the confounding problem of endometrial biopsy stimulating luteolysis, creating a control and treatment group wherein

endometrial sampling was performed on the same day post-ovulation in both groups was not possible. Therefore, the comparisons presented in this study reflect mid-luteal phase endometrial conditions before luteolysis (controls) *versus* endometrial conditions following the inhibition of luteolysis, at a time presumed to be far removed from any luteolytic signal yet prior to normal cessation of spontaneously prolonged luteal activity. Comparing these results to those of others wherein endometrial sampling occurred at the time of typical luteolysis (and assuming that OXT treatment abolished this) could be very informative. Although the endometrial conditions in the 24-day prolonged CL endometrium are not comparable temporally to those of the 10–day mid-diestrus controls, they do reflect endometrial conditions stimulated by OXT to prolong diestrus. How these conditions differ from those of a presumably normal-length diestrus are important to understand as the sequelae of failed luteolysis.

As in mares with spontaneous prolonged CL during seasonal transitional to anestrus (King *et al*., 2010), P4 levels in OXT-treated mares were lower than control mares. The fact that endometrial biopsy at day 24 did not produce an immediate luteolysis as it did following biopsy on day 10, but resulted in a delayed luteal regression (6 days *versus* 3 days) indicates that the uterine environment at day 24 following OXT stimulated prolonged luteal function is not as poised for imminent luteolysis as it is on day 10 post-ovulation.

Down-regulation of endometrial *AKR1C3* and *PTGES* gene expression and increased protein expression of PTGES in OXT treated mares suggests that regulation of PTGES involved in OXT mediated luteal maintenance presumably occurs at protein rather than at transcription level. This may suggest that the mRNA signal for *AKR1C3* and *PTGES* production occurred earlier than day 24 and was on the decline by day 24, while actual production of the protein was active at this time. These results disagree with previous studies where compared with non-pregnant controls, no differences were found in endometrial AKR1C3 and PTGES protein expression in 15-day pregnant mares (Boerboom *et al*., 2004), or in *PTGES* and *AKR1C3* mRNA levels at days 8 and 12 of pregnancy (Merkl *et al*., 2010), when prolonged luteal function is physiologic. In contrast, in another study performed later in pregnancy (days 18 and 22), *PTGES* and *AKR1C3* transcription was up-regulated (Atli *et al*., 2010). Although OXT increased *in vitro* PGE2 secretion in mid-luteal phase mare endometrium (Galvão *et al*., 2013), in early pregnant mares there were no changes in uterine flushing PGE2 concentrations (Stout & Allen, 2002). In the present study, the high PTGES protein immunoexpression found in mares with prolonged luteal function may be due to a direct stimulus of OXT in endometrial cells. In several species other than the mare, binding of OXT to cell surface transmembrane OXTR, activates a downstream MAPK signaling cascade releasing

arachidonic acid, the precursor of PG, via *PTGS2* (Vrachnis *et al*., 2011). Luteal maintenance may depend on the balance between PGF2α, as a luteolytic signal, and PGE2 as a luteotrophic signal (Galvão *et al*., 2016). In bovine endometrial epithelial cells, P4 induced a higher *in vitro* basal secretion of PGE2 (+30.0%) than PGF2α (+22.2%), while OXT stimulated the production of PGE2 (9.1-fold, $P < 0.0001$) and PGF2α (6.7-fold, $P < 0.0001$) in epithelial, but not in stromal cells (Asselin *et al*., 1996).

Although superficial and glandular endometrial epithelium immunolabeling for PTGIS was found in both control and OXT treated animals, the latter group exhibited decreased transcription and increased immunoexpression. Although the existence of endometrial PTGIS proteins has been documented in others species, their role in uterine function is unknown. To the best of our knowledge, this is the first work that shows the presence of PTGIS protein in equine mid-luteal phase endometrium. As plasma P4 levels in mares with OXT-induced prolonged luteal function were lower than in control mares, and as PTGIS expression in human endometrium was negatively correlated with decreased P4 levels (Catalano *et al*., 2011), this may be a possible explanation for higher immunoexpression of PTGIS in OXT treated mares. Further studies are needed to elucidate the role of PTGIS in mare's endometrium throughout the estrous cycle.

The lower expression of *ER1* and *PGR* in OXT-treated mares with prolonged luteal function in the present work is in agreement with other studies where luteal maintenance was achieved, as during gestation. Inhibition of these receptors has been ascribed to P4 (Hartt *et al*., 2005; Ruijter-Villani *et al*., 2015). In contrast, no difference was found in mRNA expression of *ESR1* between control and OXT treated mares in the beginning of the luteal maintenance period (Keith *et al*., 2013). The decrease in ESR2 expression in OXT treated mares found in the present study contrasts with previous reports where the expression of endometrial *ESR2* mRNA did not differ throughout the estrous cycle (Silva *et al*., 2014; Ruijter-Villani *et al*., 2015) or on days 54 and 66 of pregnancy (Silva *et al*., 2014). Others reported a downregulation of ESR2 on day 7 of pregnancy (Ruijter-Villani *et al*., 2015). Since the duration of P4 dominance affects *ESR1* mRNA content in equine endometrium (McDowell *et al*., 1999), a long-term endometrial exposure to P4 may be responsible for the low *ESR1* and *ESR2* transcription found in OXT-treated mares. However, as exposure to P4 on day 7 pregnant mares was not different from control mares in Ruijter-Villani *et al*. (2015) study, we hypothesized that the decrease in ESR2 expression observed in our study may be due to an additional mechanism, other than P4, and induced by OXT.

The detection of PGR protein in luminal and glandular epithelium, with immunoexpression being the highest in the OXT-treated group differs from the results obtained in earlier studies

with prolonged luteal function in mares (Hartt *et al*., 2005; Wilsher *et al*., 2011; Silva *et al*., 2014). In diestrus and early pregnancy, PGR mRNA and protein were not detected in luminal epithelium and were lower in stroma and deeper glandular epithelium (Hartt *et al*., 2005). Furthermore, during early pregnancy, mares lost PGR in endometrial epithelium, but not in stromal cells (Wilsher *et al*., 2011; Silva *et al*., 2014). The paracrine signaling and epithelium-stroma cross-talk involved in the regulation of early pregnancy seems to be disrupted in OXT-treated mares. In humans and mice, E2 rise stimulates PGR expression in stromal cells (Large & DeMayo; 2012). This E2 regulation of PGR expression may depend on stromal *ESR1* in mouse uterine epithelium (Kurita *et al*., 2006). In addition, although ESR1 appears to be the dominant mediator of uterine reproductive functions, in the mouse uterus ESR2 acts as a modulator of *ESR1*-mediated gene transcription and is responsible for down-regulation of PGR in luminal epithelium (Weihua *et al*., 2000). In the mare, as in the mouse, decreased endometrial expression of *ESR2* and *ESR1* transcription might be responsible for the maintenance of PGR in luminal and glandular epithelium observed in OXT-treated mares.

The up-regulation of OXTR gene and protein expression present in OXT-treated mares' endometria contrasts with other studies where no differences were observed on endometrial OXT-binding capacity (Vanderwall *et al*., 2012) and on *OXTR* mRNA (Keith *et al*., 2013), between control and OXT-treated mares at the beginning of the luteal maintenance period. However, in pregnant mares, *OXTR* mRNA was slightly up regulated in equine endometrium at day 12 (Merkl *et al*., 2010), and the OXTR gene and protein in luminal and superficial glandular epithelia expression markedly increased between days 14 and 21 (Ruijter-Villani *et al*., 2015). We found protein localization of OXTR mainly in stromal cells, in all mares, although it was the strongest in OXT-group. In ruminants, an initial up-regulation of OXTR in the luminal epithelium triggers luteolysis. Binding of OXT to luminal and glandular epithelium OXTR initiates the pulsatile secretion of PGF2α and results in luteal regression (Robinson *et al*., 2001; Lee *et al*., 2013). In comparison to what has been described in ruminants, chronic OXT administration in mares might shift cell-specific expression of OXTR from luminal epithelium to stroma, leading to luteal maintenance.

Prolonged exposure of the endometrium to P4 may decrease OXTR activity through desensitization of receptors since these receptors were up-regulated in OXT treated mares in the present work, as well as in 21 day pregnant mares (Ruijter-Villani *et al*., 2015). Non-genomic action of P4 may be responsible for blocking OXT binding to OXTR with consequent inhibition of $PGF_{2\alpha}$ luteolytic pulses resulting in prolonged luteal function. In isolated ovine endometrial plasma membranes, P4 was shown to inhibit OXT binding to its receptor by either competing with OXT for its receptor site or by binding to a closely

associated protein that alters OXTR conformation (Bishop & Stormshak, 2008). Indeed, the expression of P4 receptor membrane component 1 (*PGRMC1*) in mare endometrium is high during mid-luteal phase (Gebhardt *et al*., 2012), and early pregnancy (Ruijter-Villani *et al*., 2015). Further studies are needed to investigate the presence of endometrial membrane-bound P4 receptors in OXT-induced luteostasis, as well as the direct or indirect action of OXT on luteal maintenance.

In conclusion, administration of exogenous OXT on days 7 to 14 of the estrous cycle induced luteal maintenance in 67% of the mares. The different spatial expression of OXTR and PGR in the endometrium may be a mechanism by which chronic OXT enables luteal maintenance. The observed reduction of endometrial ESR2 may be responsible for the maintenance of PGR in luminal and glandular epithelium. Also, ESR2 may have a role in attenuating the transcriptional activity of *ESR1* in mare endometrium. This study offers new knowledge on the endometrial expression of ovarian steroids and OXTR in OXT-induced luteal maintenance in the mare.

2. Neutrophil extracellular traps formation by bacteria causing endometritis in the mare

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2.1. Abstract

Besides the classical functions, neutrophils (PMN) are able to release their DNA in response to infectious stimuli forming neutrophil extracellular traps (NETs) and killing pathogens. The pathogenesis of mare endometritis is still not completely understood. Therefore, the aim was to evaluate the *in vitro* capacity of equine PMN to secrete NETs by chemical activation, or stimulated with *Streptococcus equi* subspecies *zooepidemicus* (Szoo), *Escherichia coli* (Ecoli) or *Staphylococcus capitis* (Scap) strains obtained from mares endometritis. Besides, *ex-vivo* endometrial mucus samples obtained from mares with bacterial endometritis were evaluated for the presence of NETs. Equine blood PMN were used either without or with previous stimulation by phorbol-myristate-acetate (PMA), a strong NETs inducer, for 1, 2 or 3 h. To evaluate PMN ability to produce NETs when phagocytosis was impaired, the phagocytosis inhibitor cytochalasin (Cyt) was added after PMA. After bacteria addition, subsequent one-hour incubation was as follows: (i) PMN (negative control); (ii) PMN+PMA (positive control); (iii) PMN+Cyt (iv) PMN+bacteria; (v) PMN+PMA+bacteria; (vi) PMN+Cyt+bacteria; (vii) PMN+PMA+Cyt+bacteria. NETs were visualized by DAPI and anti-histone. Immunostaining for myeloperoxidase and neutrophil elastase was also performed on *ex-vivo* samples. A 3h incubation of PMN+PMA increased NETs ($P < 0.05$). Bacteria+25nM PMA and bacteria+PMA+Cyt increased NETs ($P < 0.05$). Bacteria Szoo was the one that induced less NETs, when compared to Ecoli or Scap ($P < 0.05$). Presence of NETs in *ex-vivo* samples was confirmed in mares with endometritis. Scanning electron microscopy showed the widespread of NETs from PMN. Equine NETs are formed by smooth fibers and globules that can be aggregated in thick bundles. Formation of NETs and the subsequent entanglement of bacteria, suggest that equine NETs might be a complementary mechanism to fight some bacteria causing endometritis in the mare.

Key words: Neutrophil extracellular traps (NETs), mare, endometritis, *Streptococcus equi* subspecies *zooepidemicus*, *Escherichia coli, Staphylococcus capitis*

2.2. Introduction

In response to inflammatory stimuli, neutrophils (PMN) migrate from blood circulation and are recruited into sites of inflammation in different tissues. They are the first line of innate immune defense against the invading bacteria (Brinkmann, 2011). The PMN present in the blood stream firmly adhere to the endothelium cell barrier, cross it, and reach the tissue to the site of infection. There, chemokines released by mast cells and resident tissue macrophages activate the PMN (Souza *et al.,* 2011). It is well established that once PMN get to the infection site they will attempt to kill bacteria by the use of diverse mechanisms (Brinkmann, 2011; Souza *et al*., 2011). Even so, the exact mechanism involved in the pathophysiology of PMN hyperactivation, as the first line of defense against pathogenic bacteria, is not fully understood. The novel paradigm of innate immunity is that, besides the classical functions of extracellular release of lytic enzymes and phagocytosis, PMN by means of their enzymatic activity, surface receptors and cytoskeleton (Neeli *et al.*, 2009), also have the capacity to release their DNA in response to infectious stimuli (Brinkmann *et al.*, 2004). Thus, at the site of infection PMN form neutrophil extracellular traps (NETs) that have the ability to bind and kill pathogens outside the cell, such as bacteria (Fuchs *et al*., 2007; Pilsczek *et al*., 2010), protozoa (Guimarães-Costa *et al*., 2009; Wardini *et al*., 2010; Abdallah *et al.,* 2012), or yeast (Urban *et al*., 2006; 2009). In addition, as reviewed by Brinkmann & Zychlinsky (2012), not only infectious agents, but also many physiologic inducers of NETs formation have been reported. Reactive oxygen species (ROS) generated at the phagosomal membrane (Fuchs *et al.,* 2007), and antibody-antigen complexes (Garcia-Romo *et al.*, 2011) are among those NETs inducers. In fact, at inflammatory sites, such as in spontaneous appendicitis in humans, NETs are also abundant (Brinkmann *et al*., 2004). Interleukin-8, lipopolysaccharide and phorbol-myristate-acetate (PMA) are also *in vitro* NETs inducers (Brinkmann *et al.*, 2004). NETs are big DNA-associated molecule complexes that consist of nucleic and cytoplasmic proteins, each with strong antimicrobial and/or immunomodulating properties (Lögters *et al*., 2009). The release of NETs by PMN with their death, when stimulated by pathogens, was called NETosis and, in contrast to necrosis and apoptosis, it may mediate antimicrobial and pro-inflammatory responses (Fuchs *et al*., 2007; Brinkmann and Zychlinski, 2012). In the last stage of NETosis, the nuclear envelope of PMN ruptures and their granules decompose, allowing the chromatin to come into direct contact with most of the proteins present in the mature PMN (Brinkmann, 2011). Thus, histones and enzymes like elastase, proteinase 3, cathepsin G and myeloperoxidase, which are NETs components, are involved in microorganism killing (Weinrauch *et al*., 2002; Wartha *et al*., 2007; Brinkmann, 2011; Windt

et al., 2012; Marin-Esteban *et al.,* 2012) or may be a host mechanism to confine the infection and prevent systemic spread of the infectious agent (Beiter *et al*., 2006).

The etiology, diagnosis and pathogenesis of acute and chronic endometritis in the mare have been the subject of extensive research for a number of decades (Ferreira-Dias *et al*., 1994; Nielsen, 2005; Hoffmann *et al.* 2009; Szóstek *et al*., 2013; Woodward *et al*., 2013). Bacterial uterine infections occur in 25–60% of barren mares and inflict major pregnancy losses (LeBlanc *et al*., 2007). A number of bacteria such as *Escherichia coli,* β hemolytic *Streptococcus, Pseudomonas aeruginosa, Klebsiella* sp*., Proteus* and *Corynebacterium* among others, has been associated with equine endometritis (LeBlanc *et al.,* 2007; Wittenbrink *et al*., 2008). The presence of these microorganisms in the mare uterus is usually simultaneous with the presence of PMN in the endometrium, and with cloudy or mucoid uterine effluxes (LeBlanc *et al.,* 2007). Some of these bacteria are able to proliferate in the mare uterus, thus making infectious endometritis to prevail, while others are killed. Thus, we postulate that some mares might fight bacteria that cause endometritis by inducing NETs release. Therefore, the aim of this study was to evaluate *in vitro* the capacity of equine PMN to secrete NETs by chemical activation, or when stimulated with *Streptococcus equi* subspecies *zooepidemicus* (Szoo), *Escherichia coli* (Ecoli) or *Staphylococcus capitis* (Scap) strains obtained from mares with endometritis.

2.3. Materials and methods

2.3.1. Bacteria preparation

Bacterial strains of Szoo*,* Ecoli and Scap were isolated from uterine swabs from mares with endometritis. Isolated bacteria were identified based on their biochemical profile (Api System, BioMérieux). All isolates were preserved at -70ºC in Buffered Peptone Water broth (Merck®) containing 20% glycerol. Bacterial isolates were plated on Columbia agar plates with 5% sheep blood and were prepared at the concentration of $5x10^7$ CFU/mL in saline solution by correlating the optical density value with a standard curve. The presence of capsule was evaluated by Anthony's method, by staining the bacteria placed on a glass slide, with crystal violet for 4 to 7 minutes, followed by rinsing with 20% copper sulfate and paper blotting.

2.3.2. Isolation of neutrophils

Venous blood was obtained by venipuncture of the jugular vein of adult healthy horses (40mL/horse; Faculty of Veterinary Medicine ethical committee approved) into heparinized

tubes (monovettes®, Sarstedt). Isolation of PMN was performed according to Roberto da Costa *et al.* (2003) with some modifications as follows. Blood was distributed into centrifuge tubes and 15% v/v of 5% of dextran (Sigma-Aldrich, St. Louis, MO, USA) was added, and incubated for 10 to 15 min at 37ºC. The obtained supernatant was gently pipette over 10mL of Ficoll separating solution (density=1.077; Biochrom), and centrifuged at 468g for 30 min. The supernatant was discarded and the pellet containing the neutrophils and red blood cells (RBC) was treated with double distilled water for 1 min to lyse the RBC. Equal volume of double concentrated phosphate-buffered saline (PBS) was used to restore isotonicity. The mixture was centrifuged at 532g at 4ºC for 10min and the PMN suspended in RPMI 1640 (Sigma-Aldrich) medium, without serum. PMN cell viability was assessed by using Trypan blue dye exclusion, which was always higher than 97%. This entire procedure was carried out under sterile environment.

2.3.3. *In vitro* neutrophil extracellular traps formation

The equine PMN cell count was adjusted to a final concentration of $2x10^6$/mL in RPMI 1640 (Sigma-Aldrich), seeded on sterile 13mm round glass coverslips placed inside the wells of 24 well culture plates (Nunc, Denmark) and incubated for 1h, at 37ºC in 5% CO_2 humidified atmosphere, as described (Brinkmann *et al*., 2010). To test the capacity of horse PMN to produce NETs without bacterial stimulation, PMN were used either without previous stimulation or stimulated by 25 or 100nM PMA (Sigma-Aldrich), a strong NETs inducer, for 1, 2 or 3h. Besides, to evaluate PMN ability to produce NETs when phagocytosis was impaired, cytochalasin (Cyt, 10µg/ml; Sigma-Aldrich), a phagocytosis inhibitor, was added 15min after PMA and further incubated. Bacteria was added to horse PMN, at the concentration of $2x10^7$/mL, previously incubated with or without Cyt or PMA and further incubated for 1h, as follows: (i) PMN; (ii) PMN+PMA; (iii) PMN+Cyt (iv) PMN+bacteria; (v) PMN+PMA+bacteria; (vi) PMN+Cyt+bacteria; (vii) PMN+PMA+Cyt+bacteria. The positive control consisted of PMN that were previously stimulated for 30min with PMA, while the negative control consisted of equine PMN alone and without any treatment. Bacteria strains added to horse PMN were Szoo, Ecoli and Scap. Each treatment was performed in four replicates and each assay was run in triplicate. From each bacterium incubation, 12 samples were analyzed. After incubation, glass coverslips with the attached PMN were carefully washed three times in PBS and incubated in 0.5% Triton X-100 (Sigma-Aldrich) to permeabilize the cells. In order to fix NETs, 4% paraformaldehyde was added to the wells for 2h. Further on, they were stained with DAPI (Vectashield with DAPI; Vector Laboratories, USA) and observed under fluorescence microscope (DM5000B, Leica). Images were captured

with a digital camera (DC350F, Leica, Germany) at random. NETs filamentous structures present per microscopic field were manually counted (Wartha *et al.*, 2007). For each coverslip, ten microscopic fields were viewed at a magnification of 200x.

2.3.4. *Ex-vivo* neutrophil extracellular traps

Endometria were obtained *post mortem* from cyclic mares (n=10) at a local abattoir. The mares were euthanized after stunning according to the European Legislation concerning welfare aspects of animal stunning and killing methods (EFSA, AHAW/04-027) and to the Portuguese legislation (DL 98/96, Art. 1º), and approved by the Faculty of Veterinary Medicine Ethics Committee.

Uterine horns were opened and signs of endometritis, like increased mucus production and altered surface epithelial color were searched. Uterine swabs were collected for bacteria isolation and cytology evaluation. Glass coverslips and glass slides (ref 4951 Plus, Superfrost® plus, Thermo Scientific) were placed on the endometrial surface and left for 30 minutes, in order to adhere to it (Manzenreiter *et al.*, 2012). Uterine inflammation was confirmed by cytology (presence of more than 3 PMNs per five fields at 400x magnification; LeBlanc *et al*, 2007) and infection was assessed by positive bacterial growth. Slides were washed in PBS and incubated in 0.5% Triton X-100. Further on, they were fixed in methanol for 10 min, stained with DAPI and observed under a fluorescence microscope. NETs images were captured with a digital camera.

2.3.5. Histone, myeloperoxidase (MPO) and neutrophil elastase (NE) markers assays

In another assays, DNA histone present in *in vitro* and *ex-vivo* NETs was immunostained with polyclonal anti-histone H2B (Lys 15) rabbit antibody (ref 5435S, Cell Signaling Technology, Isaza, Portugal) diluted at 1:100 in PBS and incubated overnight at 4ºC. This was followed by 1h incubation at room temperature with anti-rabbit IgG secondary antibody (A21206, Invitrogen, USA) diluted at 1:300. Further on, NETs were stained with DAPI (Vectashield) and observed under fluorescence microscope (DM5000B, Leica).

In order to visualize myeloperoxidase (MPO) and neutrophil elastase (NE), *ex-vivo* samples were incubated overnight at 4ºC with rabbit myeloperoxidase antibody (1:100; orb16003, Biorbyt, UK) and neutrophil elastase antibody (1:200; ab68672, Abcam, Cambridge, UK), respectively. After, they were incubated for 1hour with anti-rabbit IgG secondary antibody (1:300; A21206, Invitrogen, USA), stained with DAPI, and observed under a fluorescence microscope.

2.3.6. Scanning electron microscopy

Scanning electron microscopy (SEM) of NETs was performed. Neutrophils were stimulated *in vitro* with 25nM of PMA and with bacteria for 1h to induce NETs formation as described above. Coverslips from both *in vitro* and *ex-vivo* samples were fixed with 2.5% glutaraldehyde (AppliChem, Germany) in 0.1 M sodium cacodylate buffer for 2h, at 4°C. The samples were subsequently dehydrated in a graded ethanol series. Samples were dried using the critical point drying method and sputter coated with gold palladium, mounted on stubs, observed in a scanning electronic microscope (SEM; JEOL5200-LV) and photographed.

2.3.7. Statistical analysis

Data regarding *in vitro* NETs count were analyzed by one-way ANOVA followed by the Bonferroni multiple comparison test (GraphPad Software version 5, San Diego, USA). Significance was defined as $P<0.05$.

2.4. Results

In the absence of PMN stimulation and when PMN were incubated alone, without bacteria (negative control; Fig. 7A) no NETs were formed. However, when PMN were stimulated with 25nM PMA, a strong NETs inducer, they were able to release their DNA, as well as their nuclear and cytoplasmic proteins (Fig. 7B-D). This was noted at all incubation times (1h, 2h, 3h), even though NETs formation was the highest after 3h incubation ($p<0.05$; Fig. 7B-D). However, PMA at 100nM induced PMN necrosis and few NETs were released. Therefore, PMA was used at 25nM in all other assays. When PMN were previously treated with PMA and further incubated with bacteria (Szoo, Ecoli, or Scap) they also produced NETs (Fig. 7E-F).

Histone marker assay was performed to confirm that the extruded material forming *in vitro* NETs was PMN derived and of nucleic origin. Thus, the positive staining of equine NETs, when in the presence of the anti-histone antibody, confirmed the presence of this major protein component of chromatin in NETs (Fig.7G).

Out of the 10 endometria obtained from mares immediately after euthanasia, 4 showed signs of endometritis and had positive cytology and bacteriology. *S. equi* spp *zooepidemicus* was isolated from two endometria, *E. coli* from one mare, while a mixed culture of *S. equi* spp *zooepidemicus* and *E. coli* was obtained from the other mare. NETs were visualized in the samples obtained from the infected endometria, regardless bacteria species (Fig. 7H-P). In *ex-vivo* samples, presence of histone, MPO and NE in NETs was confirmed (Fig. 7I, L and O).

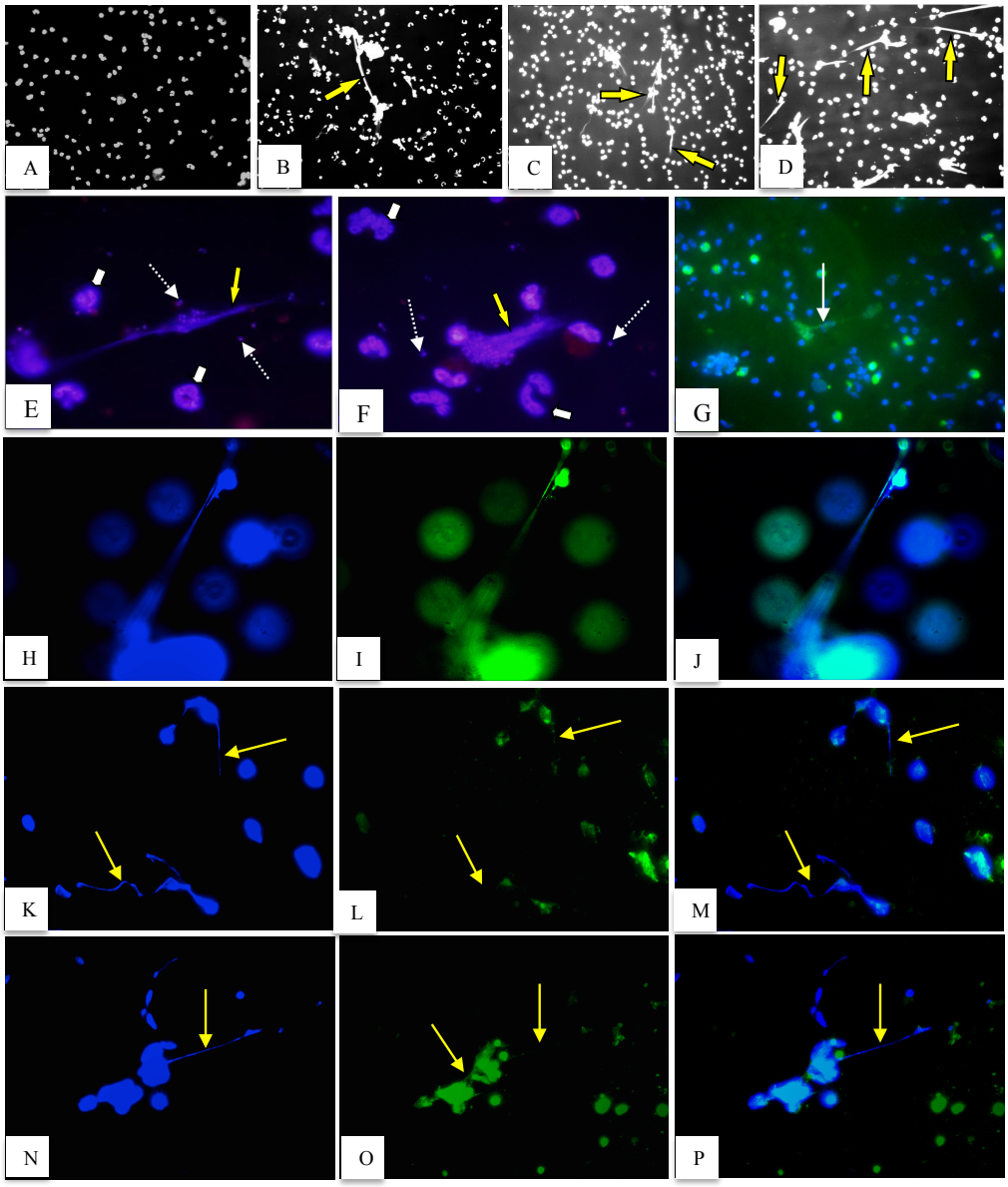

Figure 7- *In vitro* and *ex-vivo* NETs released by equine PMN. *In vitro* (A-G) and ex-vivo (H-P). NETs (yellow arrows) induced after *in vitro* PMN stimulation with PMA and mare endometritis bacteria: **A:** PMN alone (negative control); **B-D:** After 1h (B), 2h (C) or 3h (D) incubations with 25nM PMA (Mag=200x); **E:** PMN (thick arrow) stimulated with 25nM PMA and *Staphylococcus capitis* (dashed arrow); **F:** PMN stimulated with 25 nM PMA and cytochalasin, a phagocytosis inhibitor, and *S. capitis* (dashed arrow; Mag=1000x). A-F: NETs were stained with DAPI. **G**: Histone H2B positive immunostaining of equine *in vitro* NETs (white arrow) confirms the nuclear origin of extruded material (Mag=400x). **H-P:** ex-vivo NETs obtained from equine endometrium with endometritis stained with DAPI (H, K, N) and immunostaining for histone H2B (I; Mag=400x), myeloperoxidase (L; Mag=200x) and neutrophil elastase (O; Mag=200x); Right panels (J,M,P): merged.

When PMN were *in vitro* incubated with bacteria (Szoo, Ecoli, or Scap), without previous treatment with PMA, they also produced NETs (Fig. 8A-C). In fact, soon after incubation (1h), all tested bacteria were already able to induce NETs, trapping them, although Szoo was the tested microorganism that stimulated the least NETs ($p<0.05$; Fig. 8A). In contrast, Scap was the bacterium that induced the most NETs ($P < 0.05$; Fig. 8C).

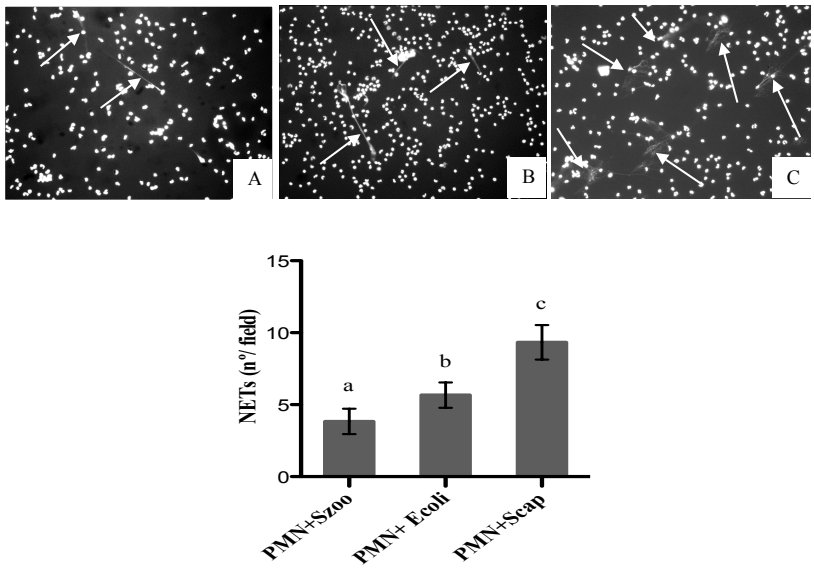

Figure 8- NETs (arrow) induced by equine PMN, without previous PMA incubation, and after 1h stimulation with *S. zooepidemicus* (Szoo; A), *E. coli* (Ecoli; B) or *S. capitis* (Scap; C). (Mag=200x). Scap was the bacteria that induced most NETs ($p<0.05$). Bars represent mean±SEM. Different letters indicate significant differences ($P < 0.05$). A-C: NETs stained with DAPI.

An increase in NETs was observed when bacteria were incubated with PMN previously stimulated by PMA, in comparison to PMA treated PMN incubated without bacteria ($P < 0.05$; Fig. 9). Nevertheless, bacteria phagocytosis inhibition by cytochalasin did not contribute for a raise in NETs formation (Fig. 9). All the bacterial strains used in these experiments were non-encapsulated as evaluated by Anthony's method.

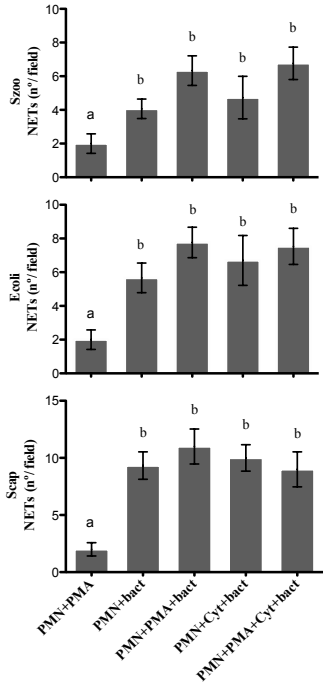

Figure 9- NETs release by horse neutrophils when incubated with PMA alone or with bacteria *S. zooepidemicus* (Szoo), *E. coli* (Ecoli) or *S. capitis* (Scap) in the presence or absence of cytochalasin, a phagocytosis inhibitor. Bars represent mean±SEM. Different letters indicate significant differences ($P < 0.05$).

Upon stimulation by 25nM PMA, the PMN flattened, made protrusions, depicted granules and formed NETs, as confirmed by scanning electron microscopy (SEM; Fig. 10 A-B).

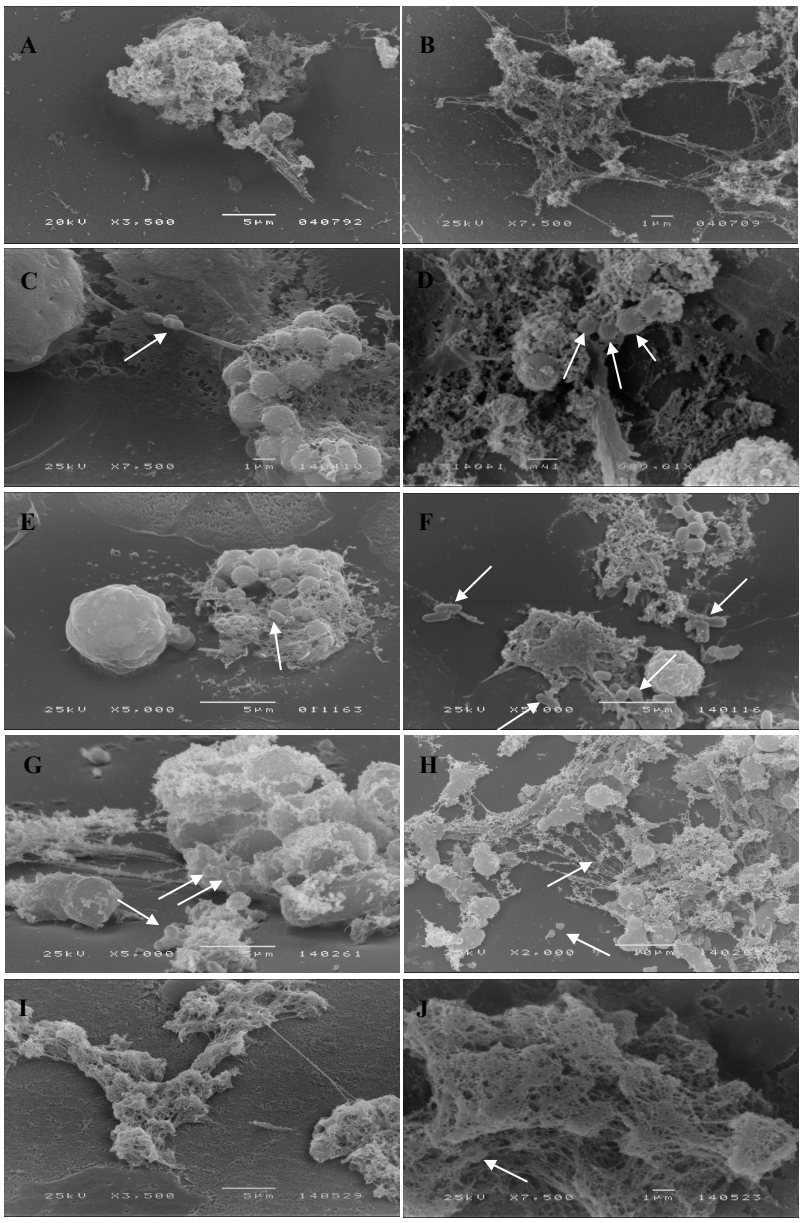

Figure 10- Scanning electron microscopy of the interaction between equine neutrophils and bacteria that cause mare endometritis. Neutrophils were incubated with: **A-B**- PMA (positive control); **C-D**- *Streptococcus equi* ssp. *zooepidemicus*; **E-F**- *Escherichia coli*; **G-H**- *Staphylococcus capitis*; **A, C, E and G-** Ultrastructure of initial phases of NETs release by PMNs that depicts many membrane protrusions with extrusion of smooth fibers and globular domains. Bacteria trapped by fibers and neutrophil granules are already visualized in C, E and G (arrows); **B, D, F** and **H**- The characteristic appearance of NETs, extended smooth fibers that intersect and bundle with each other resembling a three-dimensional network garnished with multiple spherical globules of different sizes is seen. Fibers organized in thick bundles are present. Bacteria attached to NET fibers (arrows) and neutrophil granules are depicted; **I** and **J**- Densely interlinked meshwork structures with a very similar appearance to *in vitro* NETs, obtained from endometrial mucus of mares with endometritis. **J.** *Escherichia coli* attached to fibers (arrow) can be observed.

The analysis of these NETs shows the widespread of NETs from PMN that suffered destruction throughout this process. NETs are formed by smooth fibers and globular domains that can be aggregated to thick bundles of fibers (Fig. 10 C-H). Besides, formation of NETs and the subsequent entanglement of Szoo, Ecoli or Scap, as well as the interaction between PMN and bacteria, showed that bacteria were trapped by NETs. *Ex-vivo* assays performed with endometrial mucus showed a densely-interlinked meshwork of fibers, which resemble *in vitro* NETs (Fig. 10 I-J).

2.5. Discussion

To the best of our knowledge this study shows for the first time that stimulated equine PMN have *in vitro* NETs formation capacity when in the presence of selected bacteria causing uterine infection in the mare, such as Ecoli, Szoo or Scap. Besides, *in vivo* capacity of NETs formation by PMN present in the uterus of mares with Szoo and Ecoli endometritis was also described as a novelty. Besides, the capacity of equine PMN to release NETs when in contact with equine spermatozoa was already reported (Alghamdi & Foster, 2005, Alghamdi *et al*., 2009). Those findings show that NETs produced by horse PMN can disarm Gram-positive and Gram-negative pathogens, as for other PMN species and bacteria (Brinkman *et al*. 2004). In the present work, even in the absence of bacteria, horse stimulated PMN with 25nM PMA had the ability to form NETs, as shown in the pig (500 nM PMA), even though at a deleterious dose for equine PMN (Bréa *et al*., 2012). In fact, the amount of NETs formation, depends on the degree of stimulation, and varies with the type and concentration of the stimulus (Brinkmann *et al.,* 2013). Nevertheless, untreated equine PMN did not release NETs, as reported for humans (Pilsczek *et al*., 2010). Besides, when activated horse PMN were incubated with cytochalasin, NETs persisted, as shown for other bacteria (Brinkman *et al*., 2004). Thus, the present study may suggest that besides the classic mechanism of phagocytosis already showed for the horse (Roberto da Costa *et al.*, 2003; Gardner *et al.*, 2007), NETosis might be an alternative and a complementary mechanism for mares to fight endometritis.

It is well established that the main microorganisms responsible for endometritis in the mare are Szoo and Ecoli, (Wittenbrink *et al*., 2008), which were the ones that induced less NETs. In contrast, Scap that is not a common equine endometritis pathogen was the bacterium that showed the highest NETs inducing capacity. In cows, when blood PMNs were stimulated by *E. coli* lipopolysaccharide or pathogenic mammary *E. coli*, an increase in NETs formation, ROS production, and a bactericidal effect were observed (Grinberg *et al*., 2008; Revelo &

Waldrom, 2012). Also, in response to a human *E. coli* strain, NETs were able to capture, immobilize, and kill bacteria (Marin-Esteban *et al*., 2012).

Whereas PMN have the ability to induce NETs formation when in the presence of microorganisms, some bacteria have developed a novel mechanism of innate immune evasion (Uchiyama *et al*., 2012). Only most recently the importance of their extracellular nucleases in virulence was uncovered, with the finding that they can disrupt the DNA matrix in NETs (Brinkmann & Zychlinsky, 2012; Derré-Bobillot *et al*., 2013). In fact, some *Streptococci*, which are opportunistic pathogens, have developed DNase virulence factors responsible for DNA-based degradation of NETs allowing the pathogens to escape extracellular killing (Uchiyama *et al*., 2012; Derré-Bobillot *et al.*, 2013). Also, *in vivo* susceptibility to polymicrobial infection has been ascribed to NETs depletion (Meng *et al*., 2012). An extracellular nuclease of *Streptococcus agalactiae*, attacks NETs, which is needed for *in vivo* full virulence expression (Derré-Bobillot *et al.*, 2013). Although *Streptococcus* polysaccharide capsule is an important virulence factor, non-encapsulated *S. pneumonia* are more resistant to extracellular human neutrophil elastase and cathepsin G-mediated killing than isogenic encapsulated bacteria (Windt *et al*., 2012). This resistance might be very important on mucosal surfaces and be the cause of mucosal infections (Windt *et al.*, 2012). Nevertheless, in an early study, a non-encapsulated strain of *Streptococcus equi* showed no resistance to phagocytosis by equine PMN, (Anzai *et al.*, 1999). Since the strain of Szoo used in this *in vitro* study was non-encapsulated, this might be related to the low NETs response observed by us. Most likely, in the mare, PMN phagocytosis is the mechanism of choice in the defense against Szoo, when compared to NETs. Besides, NETs might facilitate other immune processes, such as phagocytosis, and may play a role in disease pathogenesis (Cooper *et al*., 2013). The same rationale might hold true for the results regarding NETs induction with non-encapsulated Ecoli in the present study, since their behavior was similar to Szoo. In fact, non-encapsulated strains of *E. coli* from mastitis cows, were less resistant than encapsulated strains to phagocytosis by bovine PMN (Hill *et al*., 1983).

The level of NETs trapping, and the ability of microorganisms to degrade NETs or to resist to their antimicrobial components are likely to be determinant if bacterial infection will result in severe invasive disease (Wartha *et al.,* 2007; Berends *et al.,* 2010). In our *in vitro* study, equine PMN responded to Scap by releasing NETs, as reported for human PMN in contact with *Staphylococcus aureus* (Pilsczek *et al.*, 2010). The nuclease production by *S. aureus* promotes resistance against NET-mediated bacterial clearance and contributes to disease pathogenesis (Berends *et al*., 2010). Nevertheless, similar conclusions cannot be withdrawn from the present study with respect to Scap. Based on these data we are unable to tell if the

highest number of NETs observed after incubation of equine PMN with Scap, could be ascribed to a high NETs production or to a low capacity to degrade NETs or to resist to antimicrobial components.

Histones are basic DNA-binding proteins that organize chromatin in the cell nucleus and within NETs (Neeli & Radic, 2012). The presence of histone 2B in the NETs produced by equine PMN confirmed NETs nature. Besides, the H2B immunostaining of *ex-vivo* NETs, combined with the characteristic appearance of DNA fibers, provides evidence that the observed web-like structures in endometrial mucus, obtained from equine endometrium with endometritis, corresponded to NETs and are not fibrillar structures (Krautgartner *et al.*, 2010). In addition, the expression of myeloperoxidase and neutrophil elastase in *ex-vivo* NETs, suggest that they may have antimicrobial activity. Whereas bacteria and parasites probably are killed by histones, myeloperoxidase and neutrophil elastase (Brinkmann *et al*., 2004), their presence in equine NETs might suggest a similar role.

The *in vitro* NETs observed in the present work by SEM, resembled flattened three dimension meshworks, as described for *in vitro* induced NETs by PMA in humans (Manzenreiter *et al*., 2012). As reported for equine spermatozoa and PMN binding (Alghamdi *et al*., 2009), SEM studies showed that bacteria causing mare endometritis are ensnared by NETs showing a direct contact between NETs and bacteria. These NETs are formed by DNA smooth fibers decorated with globules that can be aggregated to thick bundles of fibers (Brinkman *et al.,* 2004), similar to those found in sputum of humans with cystic fibrosis (Manzenreiter *et al*., 2012). The spherical shape of globular fractions of NETs, also seen in the present study, may represent lipid membrane vesicles, possibly neutrophil ectosomes (Urban *et al*., 2009; Manzenreiter *et al*., 2012). The ectosomes are vesicles released by PMN, when activated *in vitro* and *in vivo*, expressing a strict selection of proteins (Gasser *et al*., 2003; Urban *et al*., 2009). In purulent crevicular exudates, in humans, no discrimination between fibrin and NETs based on morphological criteria in SEM was possible (Krautgartner *et al.*, 2010). In the present study, no DNAse treatment was performed in *ex-vivo* samples for SEM to overrule the presence of fibrin instead of NETs (Krautgartner *et al.*, 2010). However, immunostaining assays with histone, myeloperoxidase and elastase proved the existence of NETs produced *in vivo* in the uterus of mares with endometritis.

In conclusion, a phagocytosis independent mechanism to kill bacteria responsible for endometritis in the mare by means of NETs formation might be a novel approach to understand this pathologic condition. Besides, further knowledge on signaling pathways that lead to NETs release should be unraveled and might be considered for future therapeutic targets.

3. Constituents of neutrophil extracellular traps induce *in vitro* collagen formation in mare endometrium

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3.1. Abstract

Neutrophil extracellular traps (NETs) are DNA complexes carrying nuclear and cytoplasmic proteins, such as elastase (ELA), cathepsin-G (CAT) and myeloperoxidase (MPO). Mare endometrosis is a chronic degenerative process characterized by excessive collagen in endometrium. While NETs fight bacteria that cause endometritis, they may trigger endometrial fibrogenesis. The aim was to evaluate the *in vitro* effect of some NETs components on mare endometrial fibrogenesis and determine its relationship with endometrial histopathology or estrous cycle. Endometrial explants were incubated with NETs components (ELA, CAT, MPO or oxytocin). Collagen type I (COL1) protein and *COL1* and *COL3* genes transcription were evaluated in follicular and mid-luteal phases endometria (Kenney and Doig type I/IIA and IIB/III). Increased COL1 occurred with all NETs proteins, although endometrial response to each NETs protease depended on estrous cycle and/or endometrial category. Since ELA enhanced COL1 production, NETs persistence might be linked to mare endometrosis. Estrous cycle phase influenced endometrial fibrogenesis, suggesting that follicular phase conditions may favor endometrial collagen production. However, luteal phase endometria with moderate or severe pathological alterations may be also susceptible to fibrotic effects of NETs constituents. These data propose that NETs involvement in chronic endometritis in the mare may act as putative endometrial fibrogenic mediators.

Keywords: Neutrophil extracellular traps, fibrosis, endometrium, estrous cycle, endometrial pathology, mare, endometrosis

3.2. Introduction

It has been long established that polymorphonuclear neutrophils (PMN) play a pivotal role in innate immunity, by representing the first line of defense against invading pathogens (Kazzaz *et al.,* 2016; Nakazawa *et al.,* 2017). These inflammatory cells move to the infection site where they attempt to kill bacteria by the use of diverse classic mechanisms like extracellular release of antimicrobial peptides, lytic enzymes and phagocytosis (Brinkmann 2011). In addition, PMN also have the ability to release their own DNA, histones, and enzymes, including elastase (ELA), cathepsin G (CAT) and myeloperoxidase (MPO), in response to infectious stimuli forming neutrophil extracellular traps (NETs) that ensnare, bind and kill pathogens outside the cell (Brinkmann *et al.,* 2004; Fuchs *et al.,* 2007; Wardini *et al.,* 2010; Rebordão *et al.,* 2014; Pisanu *et al.,* 2015; Nakazawa *et al.,* 2017). The physical barrier created by NETs prevent further dissemination of microorganisms by ensnaring them at the infection site, thus limiting bacterial spread or locally confining tissue infection (Beiter *et al.,* 2006; O'Brien *et al.,* 2017). The majority of bacteria bound to NETs were alive when liberated by DNase treatment (Menegazzi *et al.,* 2012), indicating that NETs trap rather than kill microbes. NETs persistence and intensity (acute *versus* chronic process) can ultimately either fight disease or cause disease themselves (Brinkmann *et al.,* 2012). The extracellular presence of PMN serine-proteases in tissues may be one of the mechanisms leading to an exaggerated inflammatory response, tissue-specific injury (Saffarzadeh *et al.,* 2012), and finally to the development of fibrosis in lung (Korkmaz *et al.,* 2008; Cantin *et al.,* 2015), cardiovascular system (Rudolph *et al.,* 2010; Friedrichs *et al.,* 2012), liver (Pulli *et al.,* 2015) and kidney (Shimoda *et al.,* 2007).

Repair of damaged tissues is a fundamental biological mechanism that allows for the replacement of dead or damaged cells after injury, a process critically important for survival (Wynn, 2007; Oliveira *et al.,* 2016). However, irrespective to the initial causes, if this process becomes dysregulated or when the tissue damaging stimulus persists, it can lead to the development of a permanent fibrotic "scar" at the site of tissue injury. This is characterized by excessive accumulation of extracellular matrix (ECM) components - *e.g*., hyaluronic acid, fibronectin, proteoglycans, and interstitial collagens, mainly collagen I (COL1) (Wynn, 2007). In humans, fibrosis formation as a result of chronic inflammation has been reported in different tissues, like kidney (Torres *et al.,* 2014), lung (Cantin *et al.,* 2015), intestine, liver (Seki & Schwabe 2015; Rieder *et al.,* 2016)**,** and pancreas (Zechner *et al.,* 2014), among others. In the mare endometrium, a fibrotic condition known as endometrosis is a chronic degenerative process mainly characterized by extensive periglandular collagen fiber

deposition in the stroma. This pathology is responsible for infertility in this species (Kenney & Doig 1986; Lehmann *et al.,* 2011). Endometrosis is a very complex pathological process whose etiology is still unclear.

As fibrosis is often a consequence of chronic low-level inflammation, we have hypothesized that endometrosis might be a sequela to persistent/chronic endometritis in the mare, resulting from persistent low-grade inflammatory processes and consequent uterine influx of PMN. We have documented PMN ability to form NETs in mare endometrium when in the presence of some pathogenic bacteria (Rebordão *et al.,* 2014). Thus, we postulate that NETs may play a dual and contradictory role in mare endometrium. While NETs may have a beneficial action by fighting bacteria causing endometritis (Rebordão *et al.,* 2014), when inflammation is chronic and sustained, NETs components may trigger a harmful effect on the endometrium leading to fibrosis establishment (endometrosis). Therefore, the objective of this study was to evaluate the effect of some NETs components on mare's endometrial capacity to form fibrosis. The influence of endometrial inflammatory/fibrotic lesions and estrous cycle phase on endometrial fibrotic stimulation was also considered. Our results suggest that persistence of NETs components might be linked to the pathophysiology of mare endometrial fibrosis, since increased collagen type I production by endometrial tissues, characteristic of fibrosis was observed with all studied NETs proteins, although endometrial response to each specific NETs protease was different regarding the estrous cycle phase and/or with endometrial category. In the mare, estrous cycle phase appears to influence endometrial fibrogenesis, being the follicular phase, the phase with increased risk of developing this pathology.

3.3. Materials and methods

3.3.1. Biological samples collection and study design

Reproductive tracts and venous blood were collected at an abattoir from mature mares of unknown history during the breeding season. Animal handling and slaughter conformed to European welfare (EFSA, AHAW/04–027) and Portuguese (DL 98/96, Art. 18) mandates. Plasma was obtained from centrifugation of heparinized blood (Monovettes; Sarstedt, Numbrecht, Germany) and kept frozen at -20ºC for progesterone (P4) concentrations analysis according to previously detailed procedures (Roberto da Costa *et al.,* 2007). Estrous cycle stage of each mare was determined as follicular phase (FLP; n=10), or the mid-luteal phase (MLP; n=10) through a combination of ovarian and uterine condition as previously reported (Roberto da Costa *et al.,* 2007) and P4 concentrations. FLP was characterized by the presence of a follicle >35mm, absence of an active *corpus luteum* (CL) and P4 <1ng/mL. The MLP

was defined by a fully developed CL, follicles 15–20mm in diameter and P4 concentrations >6ng/mL. Immediately after ovary collection, uterine horns were opened and signs of endometritis, like increased mucus production and altered color of the surface epithelium were searched for. A sterile swab was used to quickly evaluate the presence of PMN and bacteria by routine Diff-Quick staining. Thus, only endometria, not presenting bacteria, and having in average less than two PMN per light microscopic field at 400 magnification (Riddle *et al.,* 2007) were considered as healthy uteri and were used. Immediately after ovary collection, endometrial samples from the uterine horn ipsilateral to the ovary presenting either a CL characteristic of MLP or a pre-ovulatory follicle, were separated from the myometrium and washed in sterile phosphate-buffered saline (PBS) with antibiotics (100 mg/mL streptomycin -S9137; Sigma, St Louis, MO, USA), 100 IU/mL penicillin (P3032; Sigma). Endometrial samples were immersed in either: (i) RNAlater (AM7020; Ambion, Applied Biosystems, Foster, CA, USA) for quantification of mRNA and protein expression; (ii) 4% buffered formaldehyde for histologic evaluation and further endometrial classification; or (iii) chilled (4ºC) Dulbecco's modified Eagle's medium (DMEM) and F-12 Ham medium (D/F medium; 1:1 (v/v); D-8900; Sigma) supplemented with 100 mg/mL streptomycin (S9137; Sigma, St Louis, MO, USA), 100 IU/mL penicillin (P3032; Sigma) and 2mg/mL amphotericin (A2942; Sigma) for *in vitro* explant culture studies.

A total of 20 equine endometria, from FLP (n=10) and MLP (n=10), were used in the present study. Each estrous cycle phase evaluated included samples from group I/IIA (n=5) and group IIB/III (n=5) endometria. In *in vitro* explant culture studies, endometrial tissues were challenged with different concentrations of ELA, MPO or CAT for 24h, as described in section 3.3.3. The concentrations of NETs proteins used in our study were chosen based on qualitative and quantitative protein composition of NETs and on the activities of those NETs proteases. ELA, CAT and MPO are the most abundant non-histone NETs proteins representing 5.84%, 2.47% and 1.01% of total NET protein content, respectively (Urban *et al.,* 2009). Concentrations used in this study were similar to those used in other *in vitro* assays, as well as similar to concentrations reported in physiologic and inflammatory conditions, as follows: ELA in chronic bronchitis, cystic fibrosis, and asthma (Voynow *et al.,* 2008); MPO range in blood: 0.1–2.5 ug/mL (Boudjeltia *et al.,* 2004); CAT - activated PMN in sites of injury or inflammation can express up to 160 ng of catalytically active CAT per 10^6 cells (Glusa *et al.,* 2001). The MPO concentrations used in our *in vitro* studies are within the range of those detected in the uterine lumen of mares (Parrilla-Hernandez *et al.,* 2014). Concentrations of each protease was determined by a prior dose response experiment (data not shown) wherein transforming growth factor beta-ß1 (TGF-β1) production by endometrial

explants (as a putative fibrotic marker) was evaluated in response to three concentrations of each protein; MPO (0.1, 0.5 or 1 μg/mL), CAT (0.1, 1 or 2.5 μg/mL) or ELA (0.1, 0.5 or 1 μg/mL). The highest dose of MPO and CAT did not lead to a further increase in TGF-β1 production. The lowest concentration of ELA did not induce a high secretion of TGF-β1. Bovine and horse skin collected at an abattoir was used as antigen and animal species positive controls in western-blot analysis. Endometria, skin and blood were transported on ice to the laboratory within 1h.

3.3.2. Endometrial histological analysis and classification

Formaldehyde-fixed endometrial sections (5 μm) were stained with hematoxylin (05-06014E; Bio-Optica) and eosin (HT1103128; Sigma-Aldrich) and examined under light microscope (Leica DM500). Endometria were classified by the extent of inflammation and/or fibrosis (Kenney & Doig, 1986), as category I, IIA, IIB or III, corresponding to minimum, mild, moderate or severe lesion of endometrial fibrosis, respectively. For the *in vitro* studies, endometrial samples were clustered into two groups; type I and IIA endometria (group I/IIA) or type IIB and III endometria (group IIB/III).

3.3.3. *In vitro* endometrial explant culture

For tissue culture, endometrial strips were cut, immersed in a Petri dish with PBS and antibiotics, washed thoroughly, and this procedure repeated 3 times. Individual pieces of endometria were blotted in filter paper, weighed, rinsed, and approximately 20-30mg placed in a single well of a sterile 24-well cell culture plate (Eppendorf, #0030 722.116) in 1 mL of cell culture medium, for 1h, at 37ºC in a humidified atmosphere (Biosafe Eco-Integra Biosciences, Chur, Switzerland; 5% CO2, 95% air) with gentle shaking (Titertek, Huntsville, AL, USA; 150 r.p.m.). Culture medium consisted of DME/F-12 Ham Medium (D/F medium; 1:1 (v/v); D-8900; Sigma) and 0.1% (w/v) bovine serum albumin (BSA; 735078; Roche Diagnostics, Mannheim, Germany), supplemented with 100μg/mL streptomycin (S9137; Sigma, St Louis, MO, USA), 100 IU/mL penicillin (P3032; Sigma) and 2mg/mL amphotericin (A2942; Sigma).

After 1h pre-incubation, medium was changed and endometrial explants were cultured for 24h with: (i) medium only (control); or (ii) elastase (A6959, AppliChem GmbH; ELA; 0.5, 1μg/mL); (iii) cathepsin (A6942, AppliChem GmbH; CAT; 0.1, 1μg/mL); (iv) myeloperoxidase (A6969, AppliChem GmbH; MPO; 0.1, 0.5μg/mL); or (v) positive control oxytocin (OXT; 10^{-7} M) (Nash *et al.,* 2008). Each treatment was performed in quadruplicate, and explants incubated under the same conditions as pre-incubation. After incubation, tissues

were maintained in cryotubes at -80ºC, with RNAlater, for mRNA level and protein determination of specific genes involved in fibrosis. Conditioned culture medium was collected and immediately used for alamarBlue® assay, and also stored at -80ºC, for further prostaglandin F2α and E_2 determinations. Prior to freezing, in order to prevent prostaglandins degradation during storage, 1% stabilizer (0.3 M ethylenediaminetetraacetic acid (EDTA) and 1% aspirin, No. A2093; Sigma) was added to tissue conditioned culture medium (Szóstek *et al.,* 2014).

3.3.4. Viability of endometrial explants

In order to confirm that the endometrial samples were still viable, metabolic viability of the explants was assessed by alamarBlue®(AB) (DAL1100; ThermoFisher Scientific) as previously described (Carranza-Torres *et al.,* 2014). Following each incubation time, each explant and its tissue culture medium were incubated for an additional 4 h with 10% AB, at 37°C. Afterwards, 100 μL were collected from each sample and transferred to a 96-well microplate. Fluorescence values were read using a fluorometer microplate reader (Synergy H1 Hybrid Reader, BioTek; Gene 5 software) at 560 nm excitation/590 nm emission wavelengths. The percentage viability was obtained by calculating the percentage of AB reduction per cell (Carranza-Torres *et al.,* 2014).

3.3.5. Hormone determinations

Circulating concentrations of P4 were assayed in duplicate and measured by a validated solid-phase Radioimmunoassay (RIA), without extraction, using a commercial kit (Coat-A-Count; Diagnostic Product Corporation, Los Angeles, CA, USA), and a Wallac (wizard 1470) counter. All samples were run in a single assay. The limit of detection of the assay was 0.02 ng/mL and the intra-assay coefficient of variation was 3.4%.

Culture medium was analyzed by direct enzyme immunoassay (EIA) for $PGF_{2\alpha}$ as previously described (Szóstek *et al.,* 2014). The standard curve for $PGF_{2\alpha}$ ranged from 0.19ng/mL to 50ng/mL and the intra- and inter-assay coefficient of variation (CV) were on average 8.5% and 10.7% respectively. Concentration of PGE_2 was determined using a Prostaglandin E_2 EIA kit (PGE high sensitivity ELISA kit; Catalog nº ADI-931-001; Enzo Life Sciences), according to the manufacturer procedures. According to the manufacturer, the detection limit of PGE_2 was 8.26 pg/mL. For PGE_2, the standard curve ranged from 0.039 pg/mL to 100 pg/mL, and the concentration at 50% binding (ED50) was 6.25 pg/mL. The intra- and inter-assay CV were 6.1% and 12.8%, respectively. Hormone concentrations in culture media were normalized for mg of endometrium.

3.3.6. Quantitative Real-Time polymerase chain reaction (qPCR)

For qPCR studies, RNA isolation and cDNA synthesis were performed as previously described (Galvão *et al.,* 2010). Briefly, RNA was extracted from endometria using the Total RNA Extraction and Purification kit (28704; Qiagen, Hilden, Germany), including a DNA-digestion step with an RNase-free DNase kit (50979254; Qiagen), according to the manufacturer's instructions. The RNA was quantified using the Nanodrop system (ND200C; Fisher Scientific, Hampton, PA, USA) and its quality assessed by visualization of *28S* and *18S* rRNA bands after electrophoresis through a 1.5% agarose gel and gel red staining (41003; Biotium, Hayward, CA, USA). Reverse transcription was performed using Reverse Transcriptase Superscript III enzyme (18080093; Invitrogen, GIBCO BRL, Carlsbad, CA, USA) from 1 mg total RNA in a 20 µL reaction volume using oligo (dT) primer (27-7858-01; GE Healthcare, Buckinghamshire, UK). Specific primers were designed, as well as the reference gene, using the Internet-based program Primer-3 (Untergasser *et al.,* 2012) and Primer Premier software (Premier Biosoft Interpairs, Palo Alto, CA, USA).

Real time PCR was used for assessment of mRNA transcription of collagen genes such as types I and III alpha collagen (*COL1*, *COL3*). The primers used in the present study are listed in Table 3. Before running the assay, the reference gene was validated. To determine the most stable internal control gene, four potential reference genes were initially considered, as follows: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), succinate dehydrogenase A complex, subunit A, flavoprotein (*SDHA*), beta-2-microglobulin (*B2M*) and ribosomal protein L32 (*RPL32*). For validation, samples from different stages of the estrous cycle or subjected to different treatments were run in parallel with the tested genes. *RPL32* was the most stable internal control gene (less than 2-fold changes between different biological conditions (Dheda *et al.,* 2004). Primer concentrations were optimized to the minimum concentration: lowest cycle threshold ratio. This was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Warrington, UK) using the default thermocycler program for all genes: a 10-min pre-incubation period at 95ºC was followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC, followed by a dissociation step (15 s at 95ºC, 30 s at 60ºC and 15 s at 95ºC). Both the target and reference genes were run simultaneously and all reactions were performed in duplicate wells on a 96-well optical reaction plate (4306737; Applied Biosystems) in 13 µL reaction volume containing 3.5 µL water, 1µL forward primer, 1 µL reverse primer, 6.5 µL Power SYBER Green Master Mix (4367659; Applied Biosystems) and 1 µL cDNA. In order to confirm specificity, all polymerase chain reaction (PCR) products were run on a 2.5% agarose gel (BIO-41025; Bioline, Luckenwalde, Germany). Data on relative mRNA quantification were then analyzed with the real-time PCR miner algorithm (Zhao & Fernald

2005), namely, after average cyclic threshold (Cq) and primer efficiency determinations for each sample, all Cq levels were related with primer efficiency level (E) using the equation [1/(1þE)Cq] (Zhao & Fernald 2005). Expression level of the target genes was normalized against that of the reference gene.

Table 3- Primer sequences used for *type I and III collagens* real time PCR analysis of mare endometrial explants.

Gene (Acession number)	Sequence 5´- 3	Amplicon (base pairs)
COL1A2 (XM_001492939.3)	Forward: CAAGGGCATTAGGGGACACA	196
	Reverse: ACCCACACTTCCATCGCTTC	
COL3A1 (AF117954.1)	Forward: CAAAGGAGAGCCAGGAGCAC	98
	Reverse: CTCCAGGCGAACCATCTTTG	
RPL32 (XM_001492042.6)	Forward: AGCCATCTACTCGGCGTCA	144
	Reverse: GTCAATGCCTCTGGGTTTCC	

COL1A2- collagen, type I, alpha 2; *COL3A1*- collagen, type III, alpha 1; *RPL32*- ribosomal protein L32.

3.3.7. Western blot analysis

Protein expression of type I collagen fibers was evaluated by western blotting on equine endometrial tissue explants after incubation. Even though a number of different antibodies for COL3 was tested, we were unable to detect type III collagen protein in mare endometrium. Since the primary antibody used in immunoblotting was raised against collagen type 1 of bovine origin, samples of bovine and horse skin were used as antigen and animal species positive controls, respectively. Endometrium and skin samples were minced and placed in ice-cold RIPA buffer (50 mM Tris-Hcl, pH 8; 150 mM NaCl; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulphate) with protease inhibitors (Complete Mini Protease Inhibitor Cocktail Tablets, Roche) and disrupted using mechanical (TissueLyser, Qiagen GmbH; Hilden, Germany) and ultrasonic homogenizer (Bandelin Sonopuls; Berlin, Germany). Protein was extracted and its concentration determined with Bradford reagent (500-0006; Bio-Rad, Hercules, CA, USA) (Ferreira-Dias *et al.,* 2007). Protein (40 µg) was then loaded onto 8% acrylamide gel (161-0155; Bio-Rad, Hercules, CA, USA), and transferred to nitrocellulose membranes (Ref. 10401396, Whatman; GE Healthcare Europe GmbH, Lisbon, Portugal). Protein levels were assessed using diluted specific primary antibody against collagen type I (20121; Novotec, Lyon, France; 1:1000 diluted). After overnight membrane incubation at 4ºC with the primary antibody, the density of samples on multiple gels was analyzed. A standard sample (40 µg total protein of a mix of incubated endometria samples) was loaded in a single lane of each gel in all gels (Miller 2010). This standard sample was used to normalize all bands on that same blot, and then compare them

across multiple blots. To normalize for protein loading, a mouse monoclonal antibody against β-actin (A5441; Sigma; RRID: AB_476744) was used at a dilution if 1:10,000 and incubated for 2 h, at room temperature. Membranes were incubated for 1.5 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit (1:20,000 dilution; P0448; DakoCytomation, Carpinteria, CA, USA). In the case of β-actin, the secondary antibody was HRP-conjugated goat anti-mouse (1:5,000 dilution; A2554; Sigma). After 1h incubation at room temperature, protein expression was visualized using luminol enhanced chemiluminescence (Super Signal West Pico, 34077; Thermo Scientific, Waltham, MA, USA). Chemiluminescence detection and image acquisition was performed by ChemiDoc XRS+ system (Bio-Rad Laboratories, Inc.). Densitometry signal evaluation was assessed using Image Lab^{TM} software. Dividing the units of arbitrary densitometry by standard sample density for each band normalized target protein expression, and values were expressed in terms of Relative Density. Sample Relative Density of each lane was further divided by β-actin loading-control Relative Density for that same lane.

3.3.8. Validation of collagen type I antibody

For detecting the effects of NETs components on endometrial type I collagen protein expression, western-blot analysis under denatured and reduced conditions was conducted. Since the primary antibody used in immunoblotting was raised against collagen type I of bovine origin, one assay was performed with different concentrations of bovine skin, horse skin and horse endometrium samples, to verify that the antibody was able to detect horse type I collagen (Fig. 11). Collagen type I consists of two collagen α1 (I) and one collagen α_2 (I) polypeptide chains. In horse, predicted molecular weight of collagen alpha-1(I) chain is 126kDa (Protein Database, NCBI, 2017). In all types of tested samples a molecular weight band around 126 kDa was evident (Fig. 11) corresponding to Col1 α1 (I) polypeptide. Band intensity was visualized in a concentration-dependent fashion.

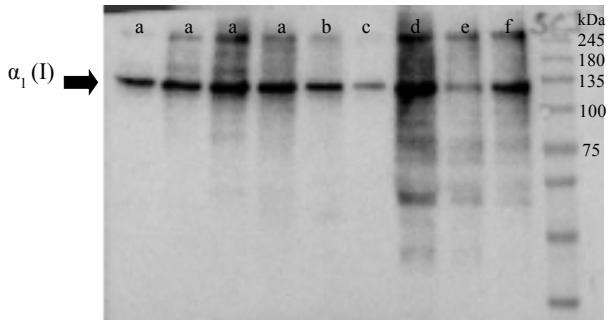

Figure 11- Collagen type I immunoblotting assay with different concentrations of bovine skin, horse skin or endometrial protein samples. Data demonstrates that the primary antibody used was able to detect horse type I collagen. Lanes: **a**- endometrial sample (40 μg); **b, c, d** -40 μg, 20 μg or 80 μg of horse skin, respectively; **e, f -** 20 μg or 40 μg of bovine skin, respectively.

3.3.9. Statistical analysis

Data are presented as mean ± S.D. Unpaired Student *t*-test was used to compare the data of endometrial explant viability and prostaglandin secretion data of non-treated endometrial explant viability. One-way analysis of variance (ANOVA) followed by Dunnett *post-hoc* test was used to compare the results between control and treated explants. Two-way ANOVA, followed by Bonferroni multiple comparison test among means, was used to determine whether any change was the result of the interaction between the type of treatment and (i) group of endometrium categories or (ii) estrous cycle phase. Statistical analyses were performed using GraphPAD PRISM (Version 5.00, GraphPad Software, San Diego, CA, USA). Results were considered significant at $P < 0.05$.

3.4. Results

3.4.1. Viability of Endometrial Explants

Endometrial explants cultured for 24h maintained high metabolic activity throughout the incubation period. No significant difference in mean viability was observed between fresh tissues (non-incubated-0h; 98.1± 1.2%) and after incubation (94.94± 3.9%); ($P > 0.05$). Explants were also capable of maintaining their secretory capacity throughout the incubation period, by producing PGF2α and PGE2 into the culture medium. PGF2α and PGE2 secretion of non-treated endometria after 24h incubation was 4.4±2.7 ng/mg endometria and 5.6±2.9 pg/mg, respectively. Those values increased in endometrial explants challenged for 24h with OXT (PGF2α: 9.882 ± 4.6 ng/mg; PGE2: 13.65±5.6 pg/mg) ($P < 0.05$). These results were independent of estrous cycle phase (data not shown). The initial pink color appearance of the explants and the secretion of PG throughout the 24h experimental period may be taken as a sign of their viability (Nash *et al.,* 2008).

3.4.2. NETs components induce high endometrial collagen expression

To determine if NETs components could have a stimulatory role in collagen deposition on mare endometrium and if that effect could be dependent on the presence of endometrial inflammatory lesions and/or on estrous cycle phase, collagen type I protein (COL1) and collagen genes transcription were evaluated in type I/IIA and IIB/III endometrial explants obtained during FLP and MLP.

Increased COL1 production resulted after incubation of FLP endometrium with the highest concentration of ELA or MPO and both doses of CAT ($P<0.05$; Fig. 12.1; 12.2). These effects were observed regardless of endometrium classification (Fig. 12.1; 12.2). Collagen type I

gene transcription in FLP type I/IIA endometria was unchanged (P > 0.05) after incubation regardless of the type or concentrations of NETs proteins (Fig. 12.3). However, in type IIB/III tissues obtained in the FLP, up-regulation of *COL1* occurred after incubation with the highest concentration of ELA ($P < 0.05$; Fig. 12.4).

Endometria obtained in the FLP responded to NETs proteases by enhancing *COL3* transcripts. In FLP type I/IIA, an up-regulation of *COL3* was observed with MPO (0.5 µg/mL, $P < 0.05$) and CAT (0.1 µg/mL, $P < 0.001$; Fig. 12.5). Likewise, in FLP type IIB/III tissues, *COL3* gene transcription increased with all concentrations of both ELA (0.5 µg/mL, $P < 0.001$; 1 µg/mL, $P < 0.01$) and CAT (0.1 µg/mL, $P < 0.01$; 1 µg/mL, $P < 0.05$) (Fig. 12.6).

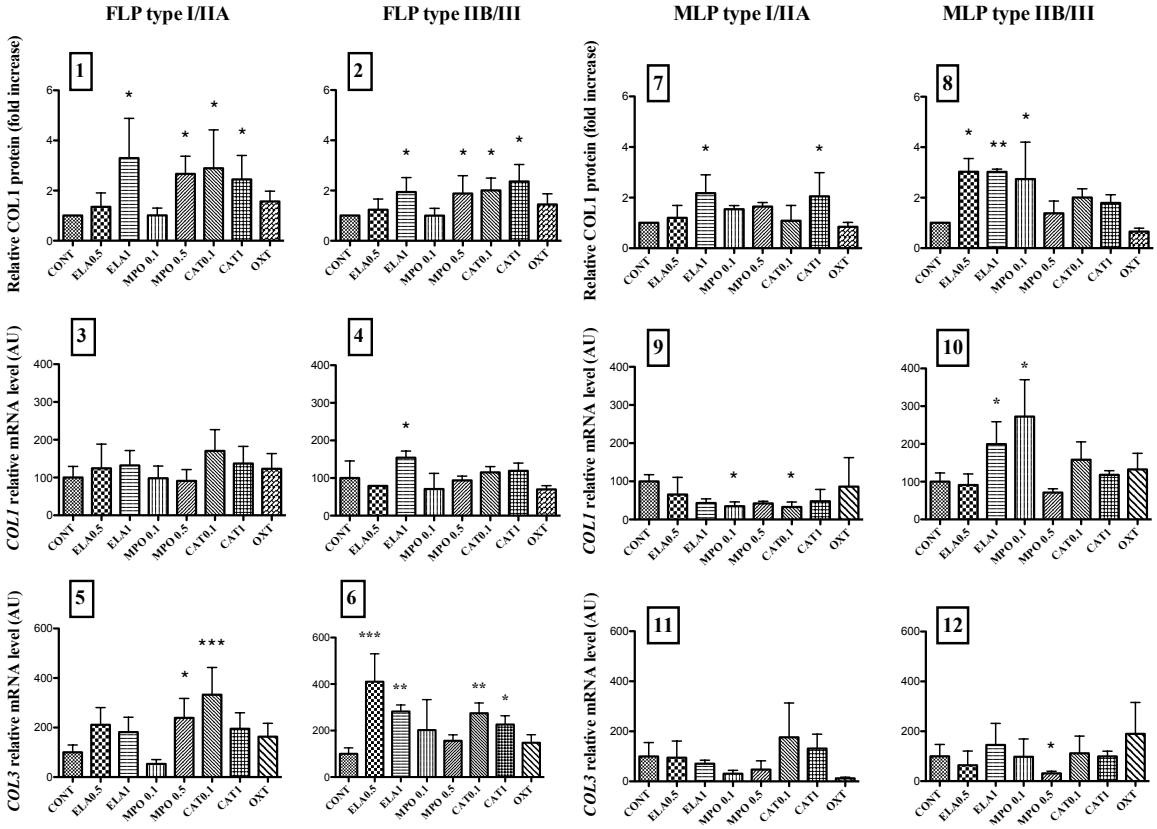

Figure 12- Relative collagen type I (COL1) protein (1, 2, 7 ,8) and transcription of *COL1* (3, 4, 9 ,10) or *COL3* (5, 6, 11 ,12) genes, by type I/IIA or IIB/III follicular or mid-luteal phase endometrial explants after incubation with different concentrations of NETs components. N=5 samples from each group- I/IIA and IIB/III, for each estrous cycle phase. Bars represent mean±S.D. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). CAT- cathepsin G (0.1 or 1 µg/mL); CONT- non-treated tissues; ELA- elastase (0.5 or 1 µg/mL); FLP- follicular phase; MLP- mid-luteal phase; MPO- myeloperoxidase (0.1 or 0.5 µg/mL); OXT- oxytocin.

Representative COL1 western blot images in FLP type I/IIA and type IIB/III endometria are shown (Fig. 13.A; 13.B).

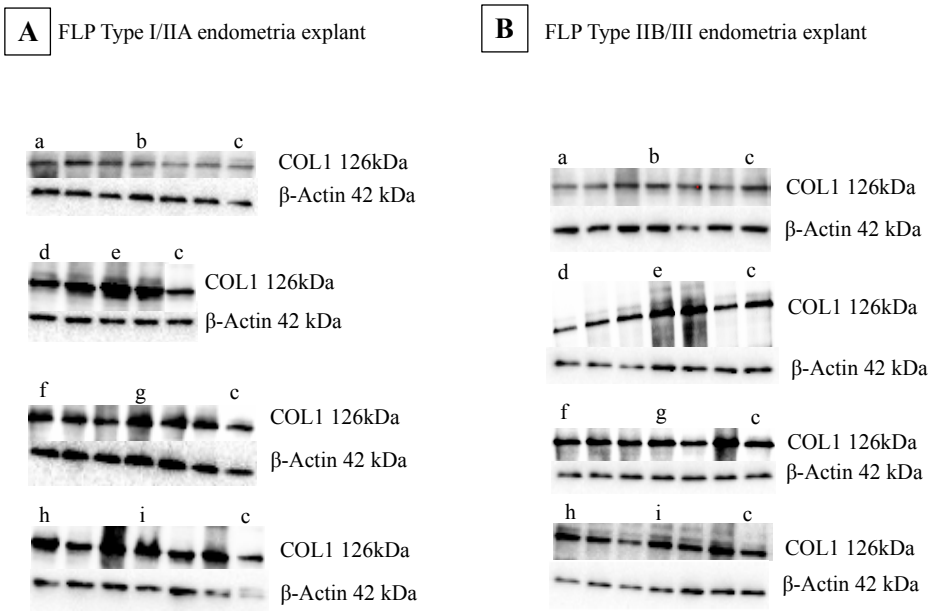

Figure 13- Panels representative of type I collagen western blotting in follicular phase. **A**- in type I/IIA endometria explants (n=5); **B**- in type IIB/III endometria explants (n=5). A standard protein (c) was used to compare the density of samples on multiple gels. Data were normalized against β-actin density values. **a**- CONT 24h; **b**- OXT 24h; **c**- standard protein; **d**- ELA 0.5 µg/mL 24h; **e**- ELA 1 µg/mL 24h; **f**- MPO 0.1 µg/mL 24h; **g**- MPO 0.5 µg/mL 24h; **h**- CAT 0.1 µg/mL 24h; **i**- CAT 1 µg/mL 24h. COL1- type I collagen; FLP- follicular phase.

During the MLP, type I/IIA endometrial explants incubated with 1µg/mL of ELA or CAT induced COL1 protein expression ($P < 0.05$; Fig. 12.7). In type IIB/III endometria, COL1 protein was elevated in explants subjected to all concentrations of ELA (0.5 µg/mL- $P < 0.05$; 1 µg/mL- $P < 0.01$) and low MPO doses ($P < 0.05$; Fig. 12.8).

Collagen gene transcription in type I/IIA MLP endometria, responded to incubation with the lowest concentration of MPO (0.1 µg/mL) and CAT (0.1 µg/mL) by down-regulating *COL1* ($P < 0.05$; Fig. 12.9). In contrast, in the type IIB/III explants exposed to the same concentration of MPO and to the highest tested dose of ELA, *COL1* was up-regulated ($P < 0.05$; Fig. 12.10). However, despite no differences in *COL3* transcripts by type I/IIA endometria ($P > 0.05$; Fig. 12.11), down-regulation of *COL3* gene transcription occurred after incubation of type IIB/III tissues with the highest concentration of MPO ($P < 0.05$; Fig. 12.12). Representative COL1 immunoblotting images in MLP type I/IIA and type IIB/IIB endometrial explants are shown in Fig. 14.A and 14.B.

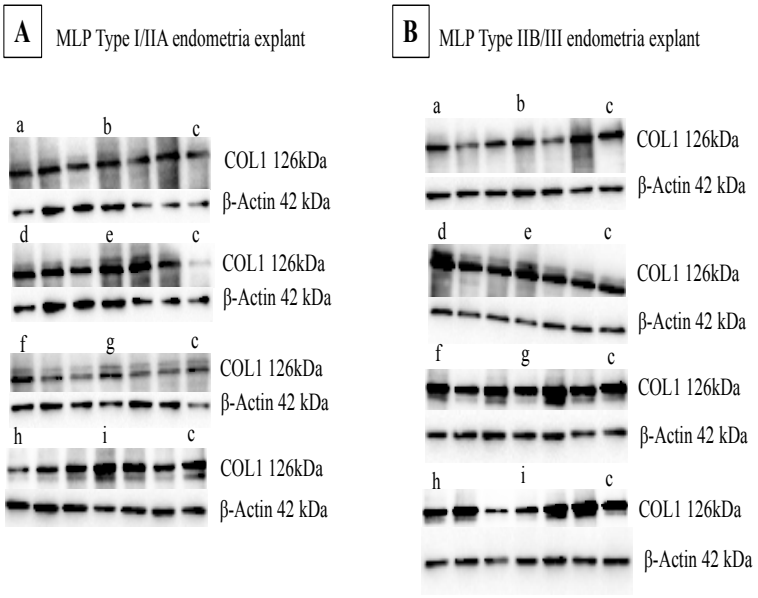

Figure 14- Panels representative of type I collagen western blotting in mid-luteal phase. **A**- in type I/IIA endometria explants (n=5); **B**- in type IIB/III endometria explants (n=5). A standard protein (c) was used to compare the density of samples on multiple gels. Data were normalized against β-actin density values. **a**- CONT 24h; **b**- OXT 24h; **c**- standard protein; **d**- ELA 0.5 μg/mL 24h; **e**- ELA 1 μg/mL 24h; **f**- MPO 0.1 μg/mL 24h; **g**- MPO 0.5 μg/mL 24h; **h**- CAT 0.1 μg/mL 24h; **i**- CAT 1 μg/mL 24h. COL1- type I collagen; MLP- mid-luteal phase.

3.4.2.1. Endometrium classification affected collagen expression

Comparison between collagen expression by endometrial category (I/IIA *vs.* IIB/III) obtained in the FLP, showed no differences in COL1 production and *COL1* gene expression, throughout the experimental period (data not shown). In contrast, in MLP endometria, comparison of fibrosis indicators between groups I/IIA *vs.* IIB/III, showed higher COL1 production in IIB/III tissues compared with I/IIA after incubation with the lowest concentrations of ELA ($P < 0.01$) or MPO ($P < 0.05$) (Fig 15.1). Transcription of the *COL1* gene was also up-regulated in the IIB/III group compared with I/IIA when endometrial explants were subjected to ELA (1μg/mL, $P < 0.05$) or MPO (0.1μg/mL; $P < 0.001$) (Fig. 15.2). No effect of endometrium category ($P > 0.05$) in *COL3* gene expression was seen (data not shown).

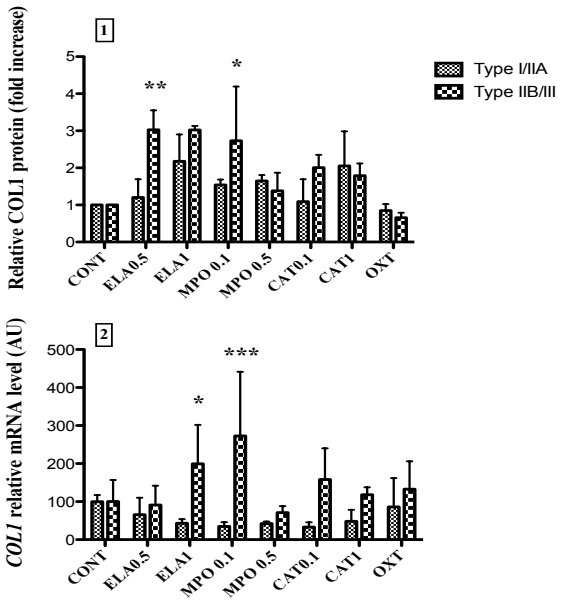

Figure 15- Effect of endometrial category (I/IIA *vs.* IIB/III) on collagen type I protein (COL1) or *COL1* gene expression by mid-luteal phase endometrial explants incubated with different concentrations of NETs components. N=5 samples from each group- I/IIA and IIB/III in MLP. Bars represent mean±S.D. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). CAT- cathepsin G (0.1 or 1 µg/mL); CONT- non-treated tissues; ELA- elastase (0.5 or 1 µg/mL); FLP- follicular phase; MLP- mid-luteal phase; MPO- myeloperoxidase (0.1 or 0.5 µg/mL); OXT- oxytocin.

3.4.2.2. Estrous cycle phase affected collagen expression

Comparison between endometria obtained during different estrous cycle phases (FLP *vs.* MLP), on endometrial response to fibrotic mediators revealed in FLP type I/IIA group, an increase in COL1 protein expression after CAT treatment (0.1 µg/ml) ($P < 0.05$; Fig. 16.1). Likewise, increased *COL1* mRNA transcription ($P < 0.05$; Fig. 16.2) with the lowest studied dose of CAT (0.1 µg/mL; $P < 0.001$) and the highest concentration of ELA ($P < 0.05$) was observed in FLP when compared with MLP. With respect to *COL3* gene, in FLP, a high transcription was observed with MPO (0.5µg/mL; $P < 0.01$) or CAT (0.1 µg/mL; $P < 0.05$) in treated explants (Fig. 16.3), compared to MLP.

Mid-luteal phase type IIB/III endometrial explants showed an enhanced COL1 production after incubation with ELA (0.5 µg/mL; $P < 0.001$) or with MPO (0.1 µg/mL; $P < 0.01$) (Fig.16.4), compared with the same treatments and endometria types during the FLP. Regarding *COL1* gene transcription an increase in its expression was depicted when MLP explants were incubated with the lowest studied concentration of MPO ($P < 0.01$) (Fig. 16.5) compared to FLP. Yet, with respect to *COL3* gene, increased transcription was observed in FLP type IIB/III group (Fig. 16.6) after incubation with ELA (0.5µg/mL; $P < 0.001$) or CAT (0.1 µg/mL; $P < 0.05$), when compared with the same treatments and endometria types during the MLP.

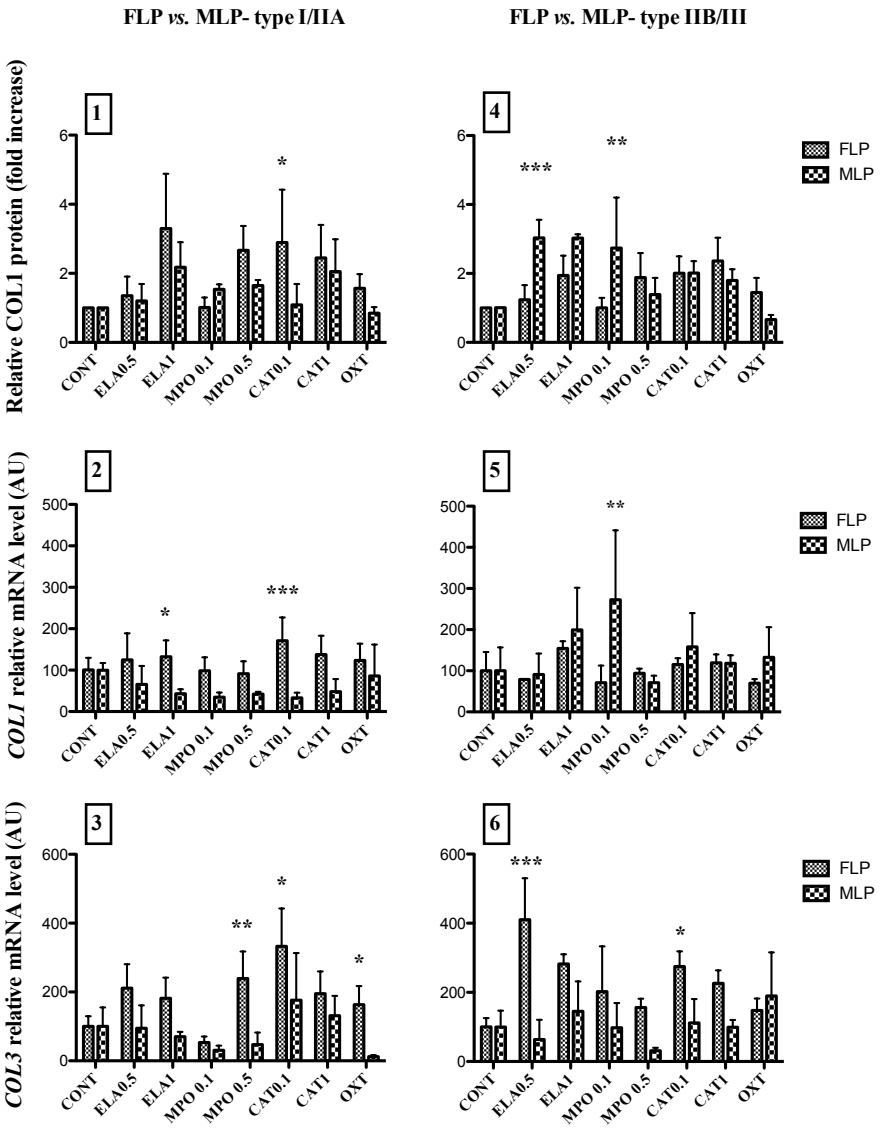

Figure 16- Effect of estrous cycle phase (follicular phase *vs.* mid-luteal phase) on collagen type I protein (COL1) (1, 4) and *COL1* (2, 5) or *COL3* (3, 6) genes expression by type I/IIA or IIA/III endometrial explants incubated with different concentrations of NETs components. N=5 samples from each group- I/IIA and IIB/III of each estrous cycle phase. Bars represent mean±S.D. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$). CAT- cathepsin G (0.1 or 1 µg/mL); CONT- non-treated tissues; ELA- elastase (0.5 or 1 µg/mL); FLP- follicular phase; MLP- mid-luteal phase; MPO- myeloperoxidase (0.1 or 0.5 µg/mL); OXT- oxytocin.

3.5. Discussion

The initiating factors of endometrosis are still uncertain and may be associated to hormonal imbalances, endometrium inflammation or be a spontaneous condition of unknown etiology, particularly in old mares (Schoon *et al.,* 1999; Hoffmann *et al.,* 2009; Reilas *et al.,* 2016; Schöniger *et al.,* 2017). To the best of our knowledge this is the first study that has

investigated the potential ability of NETs components to induce *in vitro* endometrial fibrosis by examining the expression of collagen genes and protein in mare endometrium.

In the mare endometrium, an insult like semen or pathogenic microorganisms provokes an inflammatory response, with PMN uterine infiltration that phagocytize bacteria and debris (LeBlanc & Causey 2009; Woodward *et al.,* 2013; Cadario, 2014). Previous *in vitro* studies have shown equine PMN ability to release NETs *in vit*ro, when in the presence of sperm cells (Alghamdi & Foster 2005). Also in mares with infectious endometritis, the presence of *ex-vivo* NETs decorated with ELA and MPO in uterine secretions was confirmed (Rebordão *et al.,* 2014). Besides, MPO values have been detected in uterine lumen of mares throughout the estrous cycle and they greatly increased in the presence of endometritis (Parrilla-Hernandez *et al.,* 2014). In our study, in response to MPO, endometrial explants augmented COL1 *in vitro* production, which might suggest MPO role on endometrosis establishment. Even though physiologic and pathologic concentrations of MPO in mare uterus are known (Parrilla-Hernandez *et al.,* 2014), the concentrations of ELA and CAT have not yet been reported. In susceptible mares, ELA and CAT released from peripheral blood PMN were initially lower at parturition, when compared to resistant mares, but further increased in the course of foal-heat post-breeding endometritis (Wessely-Szponder *et al.,* 2014). Since deficient uterine clearance in mare post-breeding endometritis increases the likelihood of cell damage (LeBlanc & Causey, 2009), and may result in secondary bacterial infection and PMN infiltration (Cadario, 2014), we propose that NETs persistence in the mare uterus perpetuates the inflammatory process and may lead to fibrosis development.

In endometrosis, type III collagen (predominant in healthy endometrium) is gradually replaced by type I collagen, with increasing endometrial fibrotic changes (Masseno, 2012; Costa, 2015). The exact mechanisms of excessive collagen fiber accumulation, which alter endometrial structure and results in reproductive failure, have not been elucidated. Increased collagen type I production by endometrial tissues, characteristic of fibrosis, was observed in mare endometria in the presence of all NETs proteins studied, although endometrial response to specific NETs proteases varied with the estrous cycle phase and/or with endometrial category. Our results are in agreement with those obtained by others, in which increased collagen production was observed when lung fibroblasts were treated *in vitro* with NETs (Chrysanthopoulou *et al.,* 2014). In cystic fibrosis secretions, uncontrolled NETs proteases released by PMN contribute to the destruction of lung tissue (Dubois *et al.,* 2012).

In a study on phorbol myristate acetate (PMA)-induced NETs generated by healthy human donor PMN, the dominant proteolytic activity in NETs was attributed to neutrophil ELA, but when ELA was immune-depleted, the remaining activity was attributed to CAT (O'Donoghue

et al., 2013). This is in accord with our data since among all NETs components tested, ELA (high concentration) enhanced COL1 production in all endometrial groups, regardless of estrous cycle phase. The association of ELA with fibrosis development has been demonstrated in several tissues, for example, neutrophil ELA promotes myofibroblast differentiation in lung fibrosis (Gregory *et al.,* 2015) and atheromatous plaques in cardiovascular diseases (Sharony *et al.,* 2010).

Fibrotic conditions like renal fibrosis (Shimoda *et al.,* 2007) and cystic fibrosis (Twigg *et al.,* 2015) have been associated with CAT. In our study, endometrial response to CAT profibrotic effects was dependent not only on endometrial category, but also appeared to be hormone-dependent. In type IIB/III tissues, elevated COL1 production in the follicular phase estrogen-primed endometrium was observed after incubation with CAT, whereas in MLP tissues the same effect was not seen.

Atrial (Rudolph *et al.,* 2010; Friedrichs *et al.,* 2012) and liver fibrosis (Pulli *et al.,* 2015) have been linked to MPO tissue damage. MPO-activated hepatic stellate cells, the main source of collagen production in the liver, up-regulated *in vitro* COL1 and α-smooth muscle actin myofibroblasts, via TGF-β (Pulli *et al.,* 2015). In our study, the MPO profibrotic pathway in type I/IIA endometria seems to be hormone-dependent, since enhanced COL1 production and increased *COL3* gene transcription only occurred in the FLP. However, MPO effects in type IIB/III endometria were noticed in both estrous cycle phases. It appears that fibrotic endometrium becomes independent of the uterine endocrine control mechanisms, due to ovarian steroid receptors reduction, as previously reported (Hoffmann *et al.,* 2009; Lehmann *et al.,* 2011).

The increase in COL1 output after stimulation with NETs proteins was not always associated with an increase in *COL1* gene transcription, regardless of estrous cycle phase or endometrial type. This may suggest that the mRNA signal for *COL1* production occurred before the end of the incubation period jeopardizing the detection of its raise in endometrial explants. It is known that mRNAs are less stable than proteins with a maximum half-life of approximately 7h, compared to 46h for proteins (Vogel & Marcotte 2013). Thus, protein abundance mainly depends on a dynamic balance amongst transcription, mRNA processing and damage, translation, modification and destruction of the resulting proteins (Vogel & Marcotte 2013).

In this study, we investigated whether NETs effect on collagen deposition in mare endometrium could be dependent on the presence of endometrial inflammatory lesions. During the FLP, endometrial susceptibility to NETs does not seem to be influenced by the inflammatory lesions already present, as evidenced by NETs proteases ability to stimulate COL1 production equally in type I/IIA and type IIB/III tissues. In the luteal phase, the type of

endometrium seems to influence the response to NETs. Only the highest doses of ELA and CAT caused an increase in fibrosis in tissues with minimum or mild lesions (type I/IIA), whereas in endometrium with moderate to severe lesions, low concentrations of these pro-fibrotic mediators (ELA and MPO) were sufficient to induce a fibrogenic response.

The possible hormonal influence on endometrial response to NETs proteases was addressed through evaluation of tissue response during the follicular *versus* the luteal estrous cycle phases. During the follicular phase, both types of endometria increased their transcription of the *COL3* gene when stimulated with NETs proteins. It is possible that increased transcription of this gene is accompanied by an increased production of this type of collagen fiber. However, COL3 protein production was not evaluated in this study, so this remains to be determined. Type III collagen fibers are considered to be the ones that are synthesized earlier in the development of fibrosis (Masseno, 2012; Lunelli *et al.,* 2013; Costa 2015); the ability of NETs proteins to stimulate a *COL3* reaction in all follicular endometrial types may be reflective of this. During the luteal phase, enhanced transcription of *COL3* did not occur in either endometria type. We suggest that estrous cycle may influence the type of collagen fibers that will predominate when endometrial tissue is subjected to injury. Such differences may be hormone-dependent and mediated by tissue specific catabolic or anabolic enzymes involved in collagen synthesis. Previous studies on steroid regulation of collagen synthesis in genital organs support our hypothesis. In the human uterus after estrogen (E_2) stimulation, stromal endometrial proliferation associated with extensive deposition of COL3 was observed, while P_4 stimulation caused an increase in COL3 breakdown (Stenback 1989). The ratio of type III to type I collagen in the proliferative-phase human endometrium was significantly higher than the ratios in the secretory-phase endometrium (Iwahashi *et al.,* 1996). Under the influence of high estrogen levels during late estrus, mare ovarian stroma showed an increase in COL3 while COL1 decreased. During diestrus, COL1 predominated in the stroma (Smok & Rojas 2010). Our results are in accord with Smok & Rojas (2010) suggesting that in mare reproductive tissues, E_2 may be related to COL3 production whereas P_4 appears associated with increased COL1.

Even though our *in vitro* data suggest that NETs components might contribute for endometrosis development as a follow up of PMN influx during endometritis, this might be questioned by some authors. After experimentally induced bacterial endometritis in mares, despite a transient activation of fibrotic cells in stroma, presumably caused by pro-fibrotic growth factors and cytokines released by inflammatory cells, and despite repeated inflammations, endometrosis was not exacerbated during the 2-year experimental period (Hoffmann *et al.,* 2009a). Regardless of the presence of inflammation prior to endometrial

fibrosis, a low correlation between inflammation and endometrosis grade suggests that progression of endometrium fibrogenesis in the mare, becomes independent of inflammation after a critical point (Aresu *et al.,* 2012). In contrast, in a recent study, mares with closed cervix developed a marked endometritis, with intense PMN infiltration, large fluid accumulation, and permanent pathologic endometrium changes, including fibrosis (Reilas *et al.,* 2016). The incidence of endometrosis increases in older mares, related to ovarian inactivity, and independent of parity (Ebert *et al.,* 2014; Kilgenstein *et al.,* 2015). In fact, maiden mares often have high grade endometrosis, which may be ascribed to cervical-dysfunction, poor myometrial contractility and intrauterine fluid accumulation (Pycock 2006). Therefore, even though unknown factors may be involved in endometrosis progression (Hoffmann *et al.,* 2009a), endometritis might be one of the triggers for endometrium fibrogenesis.

In conclusion, even though NETs might be a complementary mechanism to fight some bacteria responsible for endometritis in the mare (Rebordão *et al.,* 2014), they may also pose a threat to the health of the endometrium due to their protease action. Although ELA enhanced COL1 production, regardless of endometrial category or estrous cycle phase, endometrial collagen production response to each specific NETs protease depended on estrous cycle phase and/or on endometrial category. Nevertheless, further studies are required to unravel the mechanisms behind NETs protease-induced endometrial fibrosis in the mare *in vivo*.

4. Neutrophil extracellular traps proteases stimulate fibrogenic PGF2α pathway in mare endometrium

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4.1 Abstract

Endometrosis is a pathological fibrotic disease of mare endometrium that impairs uterine function, but its leading mechanisms remain largely obscure. Prostaglandins (PG), like $PGF_{2\alpha}$ play an important role in reproductive physiology and have been implicated in fibrogenesis in several organs. Signaling through their respective receptor (PGF2α-FP) mediates fibrotic actions. Persistence of neutrophil extracellular traps (NETs) may favor mare endometrial fibrogenesis, as *in vitro* endometrial explants incubation with NETs proteases induced high collagen type I production. The aim was to investigate the involvement of PGF2α pathways in collagen deposition on mare endometrium, challenged with NETs proteases. Kenney and Doig´s type I/IIA and IIB/III mare endometria, from follicular (FLP) and mid-luteal (MLP) phases, were cultured for 24h with NETs components (elastase, cathepsin-G, myeloperoxidase or oxytocin, as positive control). Expression of PGF2α protein and its synthases (*PTGS2*, *AKR1C3)* and *FP* gene transcription were evaluated. Enhanced PGF2α production and *FP* transcripts were present. Fibrogenic output was influenced by endometrial type and estrous cycle phases. In FLP and MLP type IIB/III endometria, enhanced synthesis of PGF2α or of its receptor was likely involved in mare endometrosis. Endocrine microenvironment in healthy or in pathological endometrial tissues, might modulate fibrotic $PGF_{2\alpha}$ pathway mediators, and fibrosis outcome after endometrial injury by NETs proteases.

Keywords: Neutrophil extracellular traps, fibrosis, endometrium, estrous cycle, endometrial pathology, horse, $PGF_{2\alpha}$; $PGF_{2\alpha}$ receptor

4.2. Introduction

Neutrophil extracellular traps (NETs) are big DNA-associated molecule complexes of nucleic and cytoplasmic proteins, each one of them with strong antimicrobial and/or immunomodulating/pro-inflammatory properties, responsible for bacteria killing (Fuchs *et al.*, 2007; Lögters *et al.*, 2009; Brinkmann & Zychlinski, 2012). At the end of this active process called NETosis, polymorphonuclear neutrophil (PMN) nuclear membrane ruptures and their granules decompose, allowing the chromatin to directly contact with most PMN proteins (Steinberg & Grinstein, 2007).

As a paradox, in spite of PMN being the first line of defense against the invading pathogenic bacteria, they may also be responsible for deleterious effects leading to a number of pathophysiological processes (review by Segel *et al.*, 2011). The release of proteases, such as elastase (ELA), cathepsin-G (CAT) or myeloperoxidase (MPO) may be one of the mechanisms by which PMN may contribute for a magnified inflammatory response and to tissue-specific injury with subsequent fibrosis formation (Sharoni *et al.,* 2010; Ikegame *et al.*, 2010; Segel *et al.*., 2011; Cantin *et al*., 2015; Pulli *et al*., 2015). In fact, NETs induced activation and differentiation of lung fibroblasts into myofibroblast and collagen production has been shown (Chrysanthopoulou *et al.*, 2014).

The pathogenesis of tissue fibrosis is extremely complex and regulated in the inflamed tissues by intricate interactions of pro-fibrotic cytokines, extracellular matrix (ECM) components, inflammatory cells, hormones and fibroblasts (Atamas, 2002). The cytokine transforming growth factor-β1 (TGF-β1), secreted by the inflammatory cells recruited for wound healing, is generally considered one of the most potent pro-fibrotic cytokines involved in fibrosis (Atamas, 2002; Yu *et al.*, 2003; Leask & Abraham, 2004; Wynn, 2011). In fact, TGF-β1 plays a central role in fibrogenesis through a dual effect: on fibroblast activation and fibroblast-to-myofibroblast differentiation; and on collagen production by inducing the expression of genes for ECM deposition (Atamas, 2002; Ueha *et al.*, 2012; Maroni & Davis, 2012)

Additional pathways, other than fibrotic cytokine TGF-β1, such as prostaglandins (PG), have been considered in fibrogenesis (Oga *et al.*, 2009). Prostaglandins are lipid autacoids derived from arachidonic acid (AA) that sustain homeostatic functions and mediate several pathogenic mechanisms, including the inflammatory response (Ricciotti & FitzGerald, 2011). These mediators act locally, through specific receptors, due to their extremely short half-life in blood (Oga *et al.*, 2009). Release of AA from membrane glycerophospholipids is catalyzed by phospholipase A2 (PLA2) enzymes, and AA is sequentially metabolized to prostaglandin

G2 (PGG2) and then to PGH2 by prostaglandin-endoperoxide synthase 1 (PTGS1) and/or PTGS2. PTGS1 is constitutively expressed in most tissues and responsible for housekeeping functions, while PTGS2 is regulated by several factors such as cytokines and supports sustained production of PG (Fortier *et al.*; 2008). Prostaglandin H2 is then converted to various bioactive PGs (thromboxane A2, PGD2, PGE2, PGF2α and PGI2) (Ueno *et al*., 2005). The PG produced by a given cell largely depends on the expression profile of each prostaglandin synthase enzymes (Hata & Breyer, 2004). Prostaglandin F2α is produced through the reduction of PGH2, by aldoketoreductase 1C synthases (AKR1C3) (Fortier *et al*.; 2008). Prostaglandins are ubiquitously produced and act locally in an autocrine or juxtacrine manner to elicit a diverse set of effects that modulate many physiological systems, like reproductive ones, but also have been implicated in a broad array of diseases (Hata & Breyer, 2004). Activation of a given PG receptor by its associated ligand might elicit varying responses in different cell types and tissues.

Prostaglandin F2α acting on its receptor (FP) was recently linked to fibrosis formation in other tissues, like lung (Oga *et al*., 2009; Aihara *et al*., 2013; Oga *et al.,* 2013), heart (Ding *et al*., 2012; Ding *et al*., 2014), skin (Kanno *et al.*, 2013) and synovia (Bastiaansen-Jenniskens *et al*., 2013). Nevertheless, whether TGF-β1 and PGF2α stimulate fibrogenesis through independent pathways or not has been questioned. Some authors have referred that PGF2α stimulates TGF-β1 production and plays a central role in fibrosis establishment (Hou *et al.*, 2008; Kanno *et al.*, 2013). Yet, in some systems, PGF2α-FP signaling facilitates fibrosis independently from TGF-β (Oga *et al*., 2009; Olman, 2009; Ding *et al*., 2012; Bastiaansen-Jenniskens *et al*., 2013)

Mare endometrial fibrosis (endometrosis) is the consequence of diverse insults that provoke degenerative changes of endometrial glands and surrounding stroma, characterized by periglandular arrangement of myofibroblasts and deposition of ECM, mainly collagen type I (Masseno, 2012; Costa, 2015). Knowledge of the exact mechanisms behind endometrial fibrosis is lacking. Since we have found the presence of NETs in the uterus of mares with infectious endometritis (Rebordão *et al.*, 2014), persistence of NETs may pose a threat to health of the endometria ultimately leading to fibrosis development. As profuse PG synthesis occurs in the endometrium and in other tissues that are linked to inflammatory and fibrotic conditions, we hypothesized that PG may also be involved in mare endometrial fibrosis. In our previous work, *in vitro* incubation of mare endometrial explants with some NETs proteases induced high collagen production, suggesting that NETs components could have a pathogenic role in fibrosis formation (Rebordão *et al.,* 2018). Therefore, the aim of the present study was to evaluate PGF2α pathway involvement on collagen deposition in mare

endometrium after incubation with NETs proteases, by examining the expression of PGF2α protein and its synthases and receptor gene. The influence of endometrial inflammatory/fibrotic lesions and of estrous cycle phase was also studied.

4.3. Materials and Methods

4.3.1. Collection of mare's uteri and blood and experimental design

Mare internal genitalia were collected *postmortem* from randomly designated cyclic mares, at the abattoir, during reproductive season (May-September). Animal handling and slaughter conformed to European welfare (EFSA, AHAW/04–027) and Portuguese (DL 98/96, Art. 18) mandates. Since reproductive status of the mares was unknown, blood samples were collected for progesterone (P4) determination in plasma.

Immediately after uteri collection, endometrial samples from the uterine horn ipsilateral to the ovary presenting either a growing follicle or a *corpus luteum* (CL) were carefully separated from the myometrium and washed in sterile phosphate-buffered saline (PBS) with antibiotics (100 mg/mL streptomycin- S9137; Sigma, St Louis, MO, USA and 100 IU/mL penicillin-P3032; Sigma). The macroscopic analysis of the ovaries and uteri, were used to assign a phase of the estrous cycle to each animal and to confirm the absence of signs of endometritis, such as altered surface epithelial color, increased mucus production. The presence of PMN or bacteria in uteri was evaluated microscopically on endometrial smears collected with sterile swabs and stained by Diff-Quick. The follicular phase (FLP) was characterized by the presence of a follicle >35mm diameter and the absence of an active CL, while in the mid luteal phase (MLP) a developed CL could also be associated with follicles 15–20mm in diameter (Roberto da Costa *et al.,* 2008). The healthy uteri collected were thus classified as belonging to the FLP (n=10) or to the MLP (n=10). Endometrial samples were then placed in either 4% buffered formaldehyde, for endometrial histologic evaluation and classification; RNAlater (AM7020; Ambion, Applied Biosystems, Foster, CA, USA), for quantification of mRNA expression; or kept intact in chilled (4ºC) Dulbecco's modified Eagle's medium (DMEM) and F-12 Ham medium (D/F medium; 1:1 (v/v); D-8900; Sigma) supplemented with antibiotics (100 mg/mL streptomycin, 100 IU/mL penicillin and 2mg/mL amphotericin-A2942; Sigma) for *in vitro* explant culture studies. Endometrial samples and blood were transported on ice to the laboratory within 1h.

Ten endometrial samples from each estrous cycle phase (as described in section 4.3.2; n=5 samples from each I/IIA or IIB/III group, from FLP and MLP), were further incubated with NETs proteases (see section 4.3.3). Qualitative and quantitative protein composition of NETs and the activities of those NETs proteases were the criteria used to select NETs proteins

concentrations for *in vitro* explant incubations. ELA, CAT and MPO were identified as the most abundant non-histone NETs proteins (Urban *et al.,* 2009) and their activities were between 10 nM (0.3 μg/mL) to 100 nM (3.2 μg/mL) (Dubois *et al*., 2012). Selection of NETs proteases concentration was also based on previously published reports (Voynow *et al.,* 1999; Glusa & Adam, 2001; Boudjeltia *et al.,* 2004). The MPO concentrations used in our *in vitro* studies are within the range of those detected in the uterine lumen of mares (Parrilla-Hernandez *et al.*, 2014). In addition, concentrations of each protease were determined by a preliminary dose response experiment (data not shown) performed in our laboratory, where endometrial explants TGF-β1 production (as a putative fibrotic marker) was evaluated in response to three concentrations of each protein: MPO (0.1, 0.5 or 1 μg/mL), CAT (0.1, 1 or 2.5 μg/mL) or ELA (0.1, 0.5 or 1 μg/mL). Since the highest dose of MPO and CAT did not induce a further increase in TGF-β1 production and the lowest concentration of ELA did not stimulate a high secretion of TGF-β1, further endometrial incubations with those NETs proteins were performed with the other two tested concentrations.

In our previous work, with this same experimental design and endometrial samples, we were able to assess altered collagen deposition in mare endometrium after incubation with NETs proteases **(**Rebordão *et al*., 2018). The relative fold increase of collagen type I protein by type I/IIA or IIA/III follicular phase or mid-luteal phase endometrial explants after being challenged by ELA, MPO and CAT (Rebordão *et al*., 2018), are shown in table 4.

Table 4- Relative fold increase of collagen type I protein in follicular phase and mid-luteal phase endometrial explants by categories I/IIA or IIA/III, after 24h incubation with different concentrations of NETs components, compared to their own negative controls (non-treated tissues).

		Follicular phase		Mid-luteal phase	
	μg/mL	I/IIA group (n=5)	IIB/III group (n=5)	I/IIA group (n=5)	IIB/III group (n=5)
ELA	0.5	1.46±0.28	1.09±0.21	1.29±0.24	3.02±0.30*
	1	2.96±0.79*	1.95±0.29*	2.24±0.36*	3.04±0.06**
MPO	0.1	0.99±0.15	0.96±0.15	1.50±0.07	2.73±0.84*
	0.5	2.87±0.35*	1.99±0.36*	1.61±0.08	1.28±0.28
CAT	0.1	3.18±0.76*	2.10±0.25*	1.10±0.30	1.99±0.20
	1	2.83±0.48*	2.40±0.34*	2.05±0.46*	1.84±0.19

Values represent mean±S.D. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$). ELA- elastase; MPO- myeloperoxidase; CAT- cathepsin G.

4.3.2. Mare endometrial histologic evaluation and classification

Histologic sections of 5 μm formaldehyde fixed endometrial samples were stained with hematoxylin (05-06014E; Bio-Optica) and eosin (HT1103128; Sigma-Aldrich). Each sample

was examined for the presence of inflammatory cells, periglandular fibrosis, glandular distribution and lymphatic lacunae, under light microscope (Leica DM500). Less than one PMN per field at 400x magnification (in 5 random fields) was a further basis for considering that the uteri were free of inflammation (Nielsen, 2005). Endometrial samples were classified according to Kenney & Doig (1986), in category I, IIA, IIB or III, corresponding to minimum, mild, moderate or severe lesions of endometrial fibrosis, respectively. All subsequent studies were done after clustering the endometrial samples into 2 groups, as follows: I/IIA group (included all type I and IIA endometria), and IIB/III group (comprised type IIB and type III tissues).

4.3.3. Endometrial explants and NETS components *in vitro* culture

Tissue culture was performed using strips of endometrium (n=5 samples from each group-I/IIA and IIB/III) collected from each estrous cycle phase (FLP and MLP). After being carefully washed with PBS and antibiotics (rinsed 4 times in 4 different petri dishes), small pieces of tissues were blotted in filter paper, and approximately 20-30 mg of endometria were placed in cell culture medium of each well of a sterile 24 well cell culture plate (Eppendorf, #0030 722.116), for 1h, at 37ºC in a humidified atmosphere (Biosafe Eco-Integra Biosciences, Chur, Switzerland; 5% CO2, 95% air) with gentle shaking (Titertek, Huntsville, AL, USA; 150 r.p.m.). Culture medium consisted of DME/F-12 Ham Medium (D/F medium; 1:1 (v/v); D-8900; Sigma) supplemented with bovine serum albumin (BSA; 0.1% (w/v); 735078; Roche Diagnostics, Mannheim, Germany), streptomycin (100 mg/mL), penicillin (100 IU/mL) and amphotericin (2mg/mL).

Medium was changed after 1h pre-incubation and endometrial explants were cultured for further 24h with: (i) medium – control; or (ii) elastase (ELA; 0.5, 1µg/mL); (iii) cathepsin G (CAT; 0.1, 1µg/mL); (iv) myeloperoxidase (MPO; 0.1, 0.5µg/mL); or (v) oxytocin (OXT) as positive control (10^{-7} M) (Nash *et al*., 2008). Explants were incubated under the same conditions as pre-incubation and each treatment was performed in quadruplicate. When incubation time was over, culture medium was immediately used for alamarBlue® explant metabolic viability assessment and the remaining part collected to eppendorfs with 1% of a prostaglandin stabilizer (0.3 M ethylenediaminetetraacetic acid (EDTA) and 1% aspirin, No. A2093; Sigma) and kept at -80ºC for further PGF2α determinations (Szóstek *et al.,* 2014). Conditioned explants were maintained in cryotubes with RNAlater® at -80ºC, for the assessment of specific genes involved in fibrosis.

4.3.4. Assessment of endometrial explants viability

Metabolic viability of the explants was evaluated by alamarBlue®(AB) (DAL1100; ThermoFisher Scientific), as previously explained (Carranza-Torres *et al.*, 2015). Briefly, fresh (non-incubated), treated and non-treated control explants and culture medium were incubated in the same conditions described in 2.3 section for additional 4 h with 10% AB, after 24h incubation. Fluorescence values of medium (100 μL) from each of those samples were read in duplicate against a blank (medium with AB) using a fluorometer microplate reader (Synergy H1 Hybrid Reader, BioTek; Gene 5 software) at 560 nm excitation/590 nm emission wavelengths. Percentage of endometrial explants viability was obtained by calculating the percentage (%) of AB reduction per cell (Carranza-Torres *et al.*, 2015).

In addition, since PGF2α response of non-treated and oxytocin treated explants suggests that the endometrial explants contain functional endometrial cells (Nash *et al.,* 2008) this was also used as criteria of its functionality and consequent viability.

4.3.5. Hormones assays

Plasma P4 concentrations were measured in duplicate by a validated solid-phase Radioimmunoassay (RIA), without extraction, using a commercial kit (Coat-A-Count; Diagnostic Product Corporation, Los Angeles, CA, USA), and a Wallac (wizard 1470) counter, as previously described (Rebordão *et al*., 2017a). All samples were run in a single assay. The limit of detection of the assay was 0.02 ng/mL and the intra-assay coefficient of variation for all samples was 3.4%. Circulating P4 concentrations were used to help determine estrous cycle phase of the mares. Mares with plasma P4 concentrations >6ng/mL were considered in MLP, while P4 values <1ng/mL were indicative of FLP (Roberto da Costa *et al*. 2007).

Culture medium $PGF_{2\alpha}$ was measured by direct enzyme immunoassay (EIA) as previously described (Szóstek *et al*., 2014). Standard curve for $PGF_{2\alpha}$ ranged from 0.19ng/mL to 50ng/mL and the intra- and inter-assay coefficients of variation were on average 8.5% and 10.7%, respectively. Hormone concentrations in culture media were normalized for mg of endometrium.

4.3.6. Quantitative Real-Time polymerase chain reaction (qPCR)

For qPCR studies, total RNA extraction from the endometrium was done with Total RNA Extraction and Purification kit (28704; Qiagen, Hilden, Germany), with a DNA-digestion step included (RNase-free DNase Set; 50979254; Qiagen), according to the

manufacturer instructions (Galvão *et al.,* 2010). After RNA quantification with Nanodrop system (ND200C; Fisher Scientific, Hampton, PA, USA), its quality was assessed by visualization of *28S* and *18S* rRNA bands by electrophoresis across a 1.5% agarose gel plus gel red staining (41003; Biotium, Hayward, CA, USA). Synthesis of cDNA was performed using Reverse Transcriptase Superscript III enzyme (18080093; Invitrogen, GIBCO BRL, Carlsbad, CA, USA) from 1 mg total RNA in a 20 µL reaction volume using oligo(dT) primer (27-7858-01; GE Healthcare, Buckinghamshire, UK). Internet-based program Primer-3 (Untergasser *et al*., 2012) and Primer Premier software (Premier Biosoft Interpairs, Palo Alto, CA, USA) were used to design specific primers and the reference gene, which are shown in table 5. To validate the reference gene, samples from different stages of the estrous cycle or different treatments were run in parallel for the following genes: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), succinate dehydrogenase A complex, subunit A, flavoprotein (*SDHA*), beta-2-microglobulin (*B2M*) and ribosomal protein L32 (*RPL32*). The last one was found to be the most stable internal control gene (less than 2-fold changes between different biological conditions; Dheda *et al.;* 2004).

Table 5- Primer sequences used for *AKR1C3*, *FP* and *PTGS2* real time PCR analysis of mare endometrial explant.

Gene (Acession number)	Sequence 5´- 3	Amplicon (base pairs)
AKR1C3 (XM_001500921.1)	Forward: TGGGTCACTTTCCTTCAACCA	200
	Reverse: CTTCTCCATTGCCTCCCATGT	
FP (NC_009148.2)	Forward: GTGCAATGCCATCACAGGAA	225
	Reverse: GCCATTCGGAGAGCAAACAG	
PTGS2 (GeneID: 791253)	Forward: TGCTGTTCCAACCCGTGTC	204
	Reverse: GACAATGTTCCAGACTCCCTTGA	
RPL32 (XM_001492042.6)	Forward: AGCCATCTACTCGGCGTCA	144
	Reverse: GTCAATGCCTCTGGGTTTCC	

AKR1C3- aldo-keto reductase family 1, member *C3; FP*- prostaglandin F2α receptor*; PTGS2*- prostaglandin-endoperoxide synthase; *RPL32*- ribosomal protein L32.

Primer concentrations were optimized to the minimum concentration: lowest cycle threshold ratio. Transcription of mRNA of genes involved in $PGF_{2\alpha}$ pathway, like prostaglandin endoperoxide synthase 2 (*PTGS2*), F2α synthase (*AKR1C3*-aldo-keto reductase C3), PGF2α receptor (*FP*) was assessed by Real time PCR. In StepOnePlus™ Real-Time PCR System (Applied Biosystems, Warrington, UK), the following thermocycler program was performed for all genes: 10 min pre-incubation period at 95ºC; 15 s at 95ºC for 40 cycles and 1 min at 60ºC, followed by a dissociation step (15 s at 95ºC, 30 s at 60ºC and 15 s at 95ºC). Both the target and reference genes were run simultaneously in duplicate 13 µL reaction volume (3.5 µL water, 1 µL cDNA; 1µL forward primer, 1 µL reverse primer and 6.5 µL Power SYBER

Green Master Mix- 4367659; Applied Biosystems) on a 96-well optical reaction plate (4306737; Applied Biosystems). To confirm specificity, all polymerase chain reaction (PCR) products were run on a 2.5% agarose gel (BIO-41025; Bioline, Luckenwalde, Germany). Real-time PCR miner algorithm (Zhao & Fernald 2005) was used to quantify relative mRNA data; for each sample, average cyclic threshold (Ct) was related to the primer efficiency level (E) using the equation [1/(1þE)Ct] (Zhao & Fernald 2005). Target genes expression was then normalized against that of the reference gene and relative expression values were calculated. Relative mRNA transcription of control samples was compared with treated explants data.

4.3.7. Statistical analysis

Statistical analysis of the data was performed using GraphPAD PRISM (Version 5.00, GraphPad Software, San Diego, CA, USA). Prostaglandin F2α secretion data of non-treated and oxytocin-treated endometrial explants was evaluated by Student *t*-test. Results of endometrial explants viability and data concerning relative mRNA and prostaglandin output of control and treated explants was analyzed by One-way analysis of variance (ANOVA) followed by Dunnett *post-hoc* test. To determine if any difference was due to the existence of interaction between the type of treatment and (i) group of endometrium categories; or (ii) estrous cycle phases, Two-way ANOVA, followed by Bonferroni multiple comparison test among means, was used. Data are presented as mean ± S.D. A *P* value less than 0.05 was considered statistically significant.

4.4. Results

4.4.1. Viability of Endometrial Explants

Endometrial explants showed high metabolic activity and no differences in % of AB reduction were observed between fresh (non-incubated; 98.1± 1.2%) and 24h incubated control (non-treated) tissues (94.94± 3.9%) ($P > 0.05$). After incubation of mare endometrial explants with ELA, MPO and CAT, the % of difference in AB reduction between treated and non-treated control samples was not different (ELA 0.5µg/mL= 94.62%±7.1; ELA 1µg/mL = 95.05%±7.5; MPO 0.1µg/mL= 97.79%±14.7; MPO 0.5µg/mL= 97.26%±12.3; CAT 0.1µg/mL= 95.52%±7.1; CAT 1 µg/mL= 98.7%±10.6) ($P > 0.05$). Besides, the PGF2α response of explants to oxytocin treatment suggests that the endometrial explants contain functional endometrial cells (Nash *et al.,* 2008). PGF2α secretion by endometrial explants

after incubation with OXT (9.882 ± 4.6 ng/mg) was increased when compared with incubated non-treated tissues (4.4±2.7 ng/mg) ($P < 0.05$).

4.4.2. PGF2α pathway is involved in fibrogenesis of mare endometrium

Increased collagen type I production by mare endometria, characteristic of fibrosis, in the presence of NETs proteins was observed in our previous study (Rebordão *et al*., 2018; table 4), using the same endometrial samples evaluated in this follow-up work. Thus, transcription of genes involved in endometrial PG secretory function, like prostaglandin endoperoxide synthase (*PTGS2*), *AKR1C3* and *FP,* as well as PGF2α output were studied, in order to determine if the enhanced collagen type I protein detected after incubation of endometrial tissues with NETs components (Rebordão *et al*., 2018) could be related to a change in PGF2α pathway.

4.4.2.1. Follicular phase type I/IIA mare endometrium explants

In type I/IIA mare endometrium explants obtained from FLP, increased COL1 protein and *PTGS2* transcripts ($P < 0.05$; Fig. 17.1), was observed at 24h incubation with the highest tested concentration of MPO with no further changes in the other studied parameters. No differences were found in *AKR1C3* transcripts throughout the incubation period, regardless of NETs proteins ($P > 0.05$; Fig. 17.2). Enhanced COL1 after 24h incubation with the lowest concentration of CAT was related with high transcription of *PTGS2* ($P < 0.05$; Fig. 17.1) and of *FP* gene ($P < 0.05$; Fig. 17.3). Further increase in COL1 production and PGF2α production (Fig. 17.4) was observed at 24h incubation with the highest tested concentrations ELA ($P < 0.001$) and CAT ($P < 0.05$).

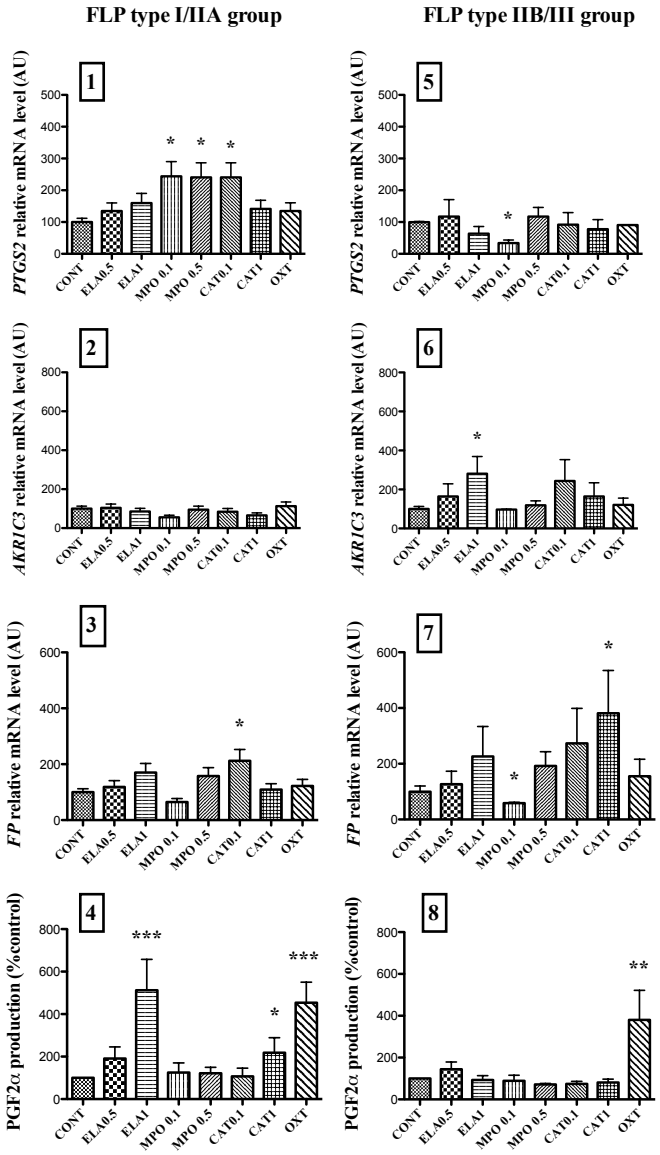

Figure 17- Transcription of prostaglandin-endoperoxide synthase 2 (*PTGS2*) (1, 5), PGF2α synthase (*AKR1C3*) (2, 6) or PGF2α receptor (*FP*) (3, 7), genes and PGF2α production (4, 8), by follicular phase endometrial explants after incubation with different concentrations of NETs components. N=5 samples from each group- I/IIA and IIB/III in FLP. Bars represent mean±S.D. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). CAT- cathepsin G (0.1 or 1 µg/mL); CONT- non-treated tissues; ELA- elastase (0.5 or 1 µg/mL); FLP- follicular phase; MPO- myeloperoxidase (0.1 or 0.5 µg/mL); OXT- oxytocin.

4.4.2.2. Follicular phase type IIB/III mare endometrium explants

In FLP type IIB/III mare endometrium explants, no changes in COL1 production, was related to decreased *PTGS2* ($P < 0.05$; Fig. 17.5) and *FP* genes transcription after incubation with the lowest concentration of MPO ($P < 0.05$; Fig. 17.7). However, tissue incubation with the highest concentration of ELA enhanced COL1 production and *AKR1C3* ($P < 0.05$; Fig. 17.6). Also, after 24h incubation with the highest concentration of CAT increased COL1 production

and *FP* transcripts ($P < 0.05$; Fig. 17.7) were noted. PGF2α production remained unchanged throughout the incubation period regardless of NETs components, but it was up-regulated with OXT ($P < 0.01$; Fig. 17.8). No differences were observed in any of PGF2α-pathway mediators when tissues were challenged with the highest doses of MPO or with the lowest of CAT, although enhanced production of COL1 was observed after 24h.

4.4.2.3. Mid-luteal phase type I/IIA mare endometrium explants

In MLP type I/IIA mare endometrium, no changes in COL1 production was related to increased *PTGS2* gene transcription ($P < 0.05$; Fig. 18.1) after incubation with both concentrations of MPO. Yet, the same type of endometrium, responded to 24h incubation with highest concentrations of ELA and CAT by up-regulating COL1 protein and down-regulating *AKR1C3* ($P < 0.05$; Fig. 18.2), and *FP* ($P < 0.05$; Fig. 18.3) genes transcription.

Decreased transcription of *AKR1C3* was also observed with the lowest concentrations of ELA and CAT ($P < 0.05$; Fig. 18.2), while down-regulation of *FP* transcripts also occurred with the lowest doses of ELA and MPO ($P < 0.05$; Fig. 18.3). None of those effects were linked to an increase in collagen production.

No differences were found in PGF2α production after 24h incubation, regardless of NETs proteins ($P > 0.05$), although it increased with OXT ($P < 0.05$; Fig. 18.4).

4.4.2.4. Mid-luteal phase type IIB/III mare endometrium explants

In MLP type IIB/III mare endometrium explants, decreased *PTGS2* transcripts were observed at 24h incubation with the highest concentration of MPO and the lowest dose of CAT with no changes in COL1 production ($P < 0.05$; Fig. 18.5). The highest concentration of ELA ($P < 0.01$) and the lowest dose of MPO ($P < 0.05$) up-regulated COL1 protein, *AKR1C3* (ELA- $P < 0.05$; MPO- $P < 0.01$) (Fig. 18.6) and *FP* (ELA- $P < 0.05$; MPO-$P < 0.001$) (Fig.18.7) gene transcription. However, increased COL1 production after 24h incubation with the lowest concentration of ELA was associated with decreased transcription of *FP* gene ($P < 0.05$; Fig. 18.7). In contrast, tissue incubation with both concentrations of CAT induced *FP* gene transcription ($P < 0.05$; Fig. 18.7), although no increase in COL1 protein was noted. No changes were seen in PGF2α output when tissues were subjected to all tested NETs proteases, although it was increased after incubation with OXT ($P < 0.05$; Fig. 18.8).

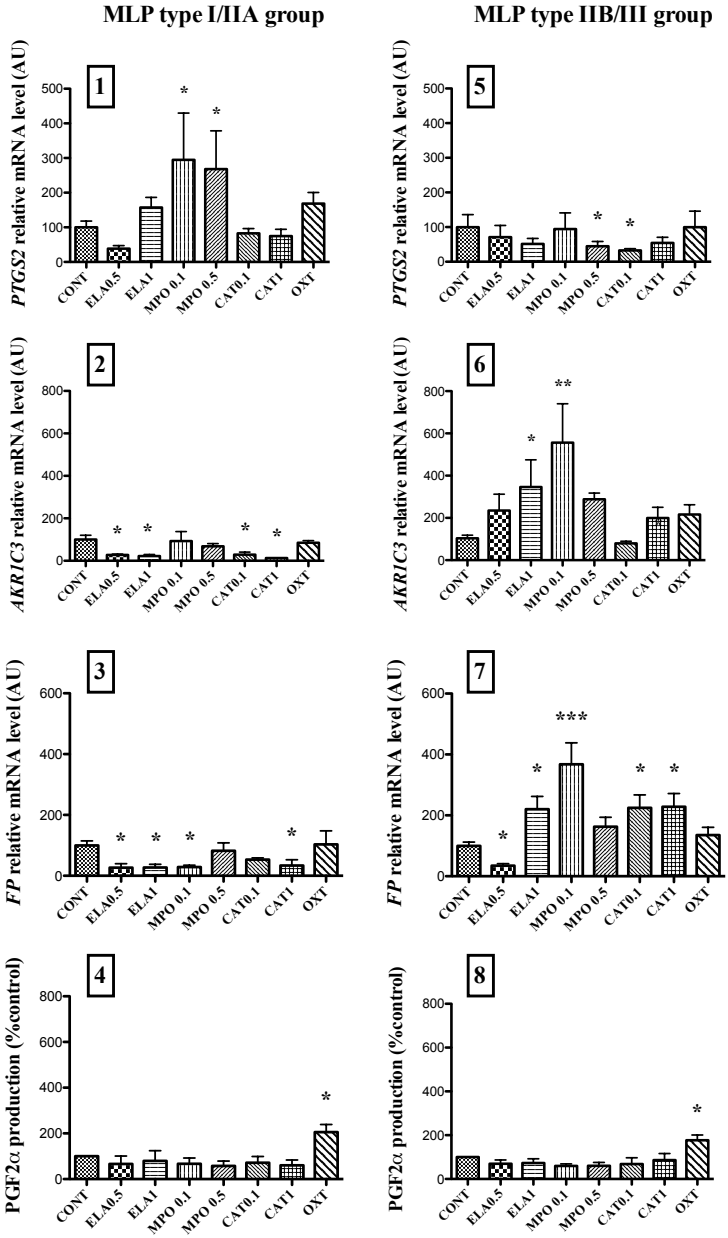

Figure 18- Transcription of prostaglandin-endoperoxide synthase 2 *(PTGS2*) (1, 5), PGF2α synthase *(AKR1C3)* (2, 6) or PGF2α receptor *(FP)* (3, 7), genes and PGF2α production (4, 8) by mid-luteal phase endometrial explants after incubation with different concentrations of NETs components. N=5 samples from each group- I/IIA and IIB/III in MLP. Bars represent mean±S.D. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). CAT- cathepsin G (0.1 or 1 µg/mL); CONT- non-treated tissues; ELA- elastase (0.5 or 1 µg/mL); MLP- mid-luteal phase; MPO- myeloperoxidase (0.1 or 0.5 µg/mL); OXT- oxytocin.

4.4.3. Endometrial category influences the putative PGF2α fibrogenic pathway

The influence of endometria type groups (I/IIA *vs.* IIB/III) on the effect of NETs components in putative PGF2α fibrogenic pathway was evaluated. This analysis was performed comparing the same treatments, within the same estrous cycle phase (FLP or MLP).

4.4.3.1. Type I/IIA *vs.* IIB/III mare endometrium in follicular phase

Type IIB/III endometrial explants obtained in FLP exhibited an increased transcription of *FP* gene after 24h incubation with the highest concentration of CAT ($P < 0.05$; Fig. 19.1) when compared to type I/IIA tissues. Higher PGF2α production was noted in type I/IIA explants incubated with the highest concentration of ELA, after 24h ($P < 0.01$; Fig. 19.2), in comparison to type IIB/III group.

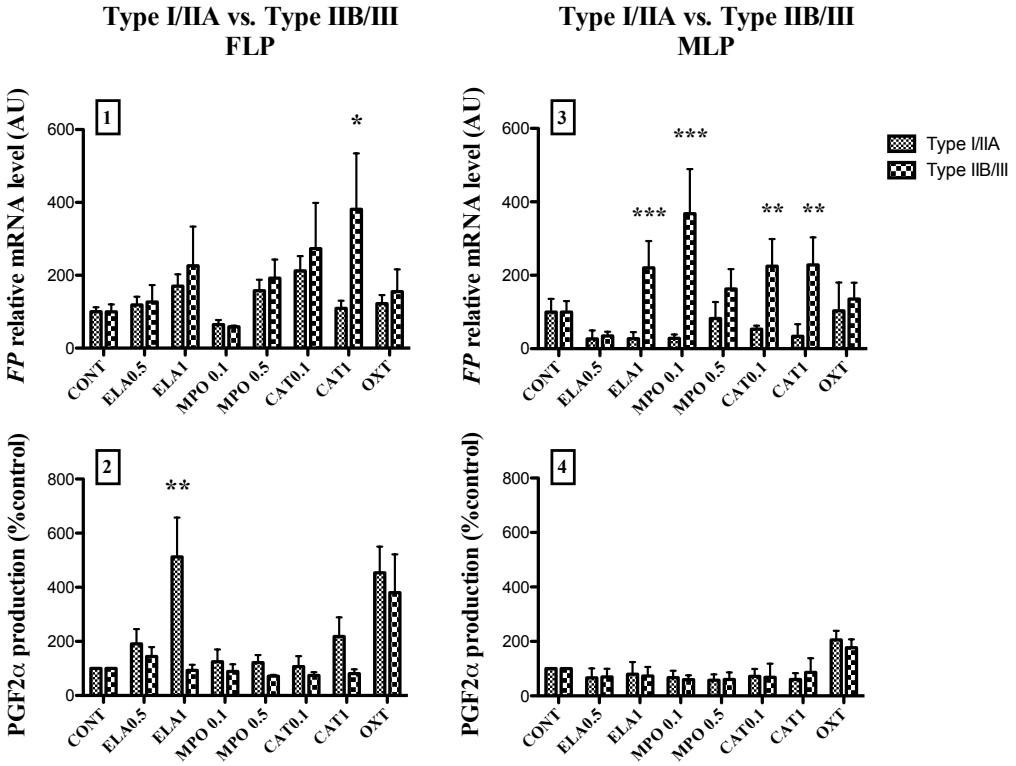

Figure 19- Effect of endometrial category (type I/IIA *vs.* type IIB/III) on PGF2α receptor *(FP)* (1, 3) gene transcription or PGF2α production (2, 4) by endometrial explants incubated with different concentrations of NETs components. N=5 samples from each group- I/IIA and IIB/III for each estrous cycle phase. Bars represent mean±S.D. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). CAT- cathepsin G (0.1 or 1 µg/mL); CONT- non-treated tissues; ELA- elastase (0.5 or 1 µg/mL); FLP- follicular phase; MLP- mid-luteal phase; MPO- myeloperoxidase (0.1 or 0.5 µg/mL); OXT- oxytocin.

The most elevated *PTGS2* gene expression was detected in I/IIA group with both tested concentrations of MPO (MPO 0.1µg/mL - $P < 0.001$; MPO 0.5µg/mL - $P < 0.05$) and the lowest dose of CAT ($P < 0.01$) after a 24h incubation (Fig. 20.1). In contrast, up-regulation of *AKR1C3* transcripts were only detected in type IIB/III endometrial explants, after incubation with the highest concentration of ELA and with the lowest dose of CAT ($P < 0.05$; Fig. 20.2).

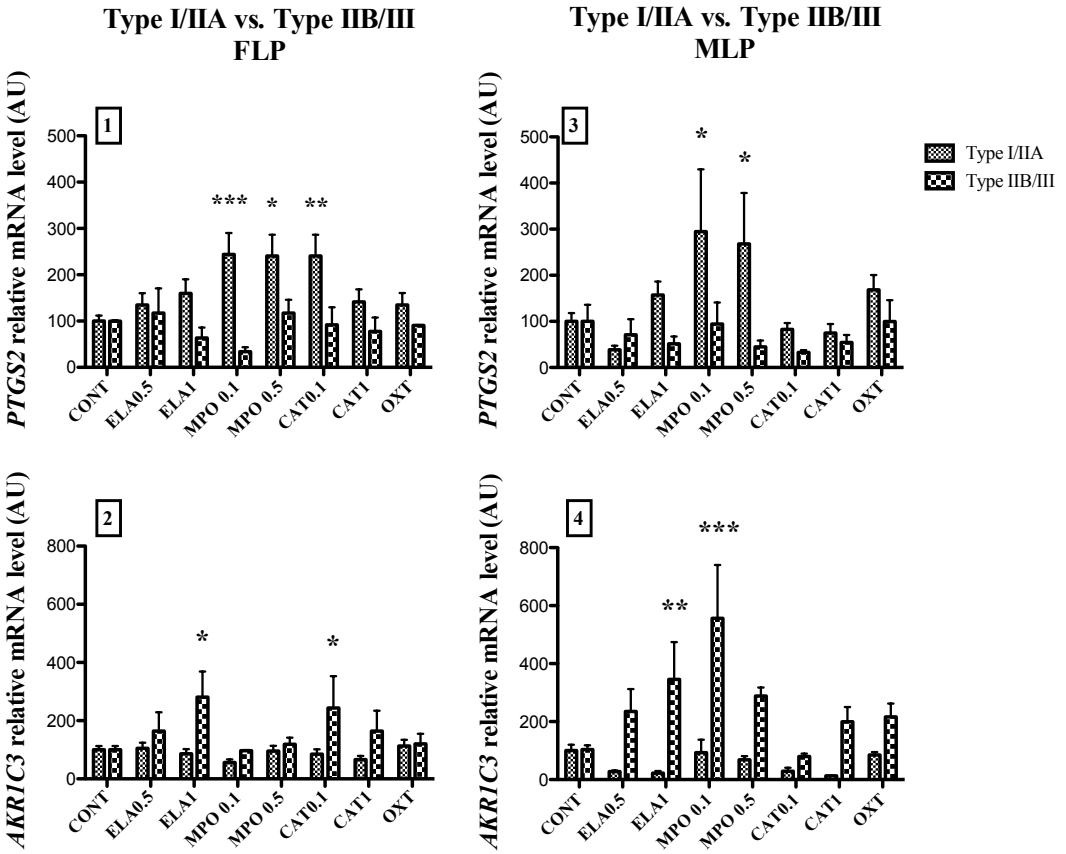

Figure 20- Effect of endometrial category (type I/IIA *vs.* type IIB/III) on the transcription of prostaglandin-endoperoxide synthase 2 (*PTGS2*) (1, 3) or PGF2α synthase (*AKR1C3*) (2, 4) genes by endometrial explants incubated with different concentrations of NETs components. N=5 samples from each group- I/IIA and IIB/III for each estrous cycle phase. Bars represent mean±S.D. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). CAT- cathepsin G (0.1 or 1 µg/mL); CONT- non-treated tissues; ELA- elastase (0.5 or 1 µg/mL); FLP- follicular phase; MLP- mid-luteal phase; MPO- myeloperoxidase (0.1 or 0.5 µg/mL); OXT- oxytocin.

4.4.3.2. Type I/IIA *vs.* IIB/III mare endometrium in mid-luteal phase

In type IIB/III endometrial explants obtained in MLP, higher *FP* transcripts resulted after incubation with the highest dose of ELA ($P < 0.001$), the lowest dose of MPO ($P < 0.001$) and both concentrations of CAT ($P < 0.01$), compared to type I/IIA tissues (Fig. 19.3). Nevertheless, no differences were detected in PGF2α production between endometrial categories in MLP explants (Fig. 19.4).

In type I/IIA endometria, after 24h incubation, greater *PTGS2* gene expression was noted with both tested concentrations of MPO ($P < 0.05$; Fig. 20.3). In contrast, higher *AKR1C3* gene transcription was only detected in type IIB/III endometrial explants, with the highest concentration of ELA ($P < 0.01$) and with the lowest MPO dose ($P < 0.001$) at 24h (Fig. 20.4), compared with type I/IIA group.

4.4.4. Estrous cycle phase affected putative fibrogenic mediators of PGF2α via

The influence of different phases of mare estrous cycle (FLP *vs.* MLP) on the effect of NETs components in putative PGF2α fibrogenic pathway was also analyzed. This evaluation was done in the same endometria type (I/IIA or IIB/III), comparing the same treatments at different incubation times.

4.4.4.1. Follicular phase *vs.* mid-luteal phase in type I/IIA endometria

Regarding *FP* gene transcription, increased levels were found in FLP explants, when compared to MLP incubated with ELA (1 μg/mL) or CAT (0.1 μg/mL) ($P < 0.001$; Fig. 21.1). A marked increase in PGF2α production was observed in FLP endometria incubated with the highest concentration of ELA, ($P < 0.001$; Fig. 21.2) compared with MLP endometria.

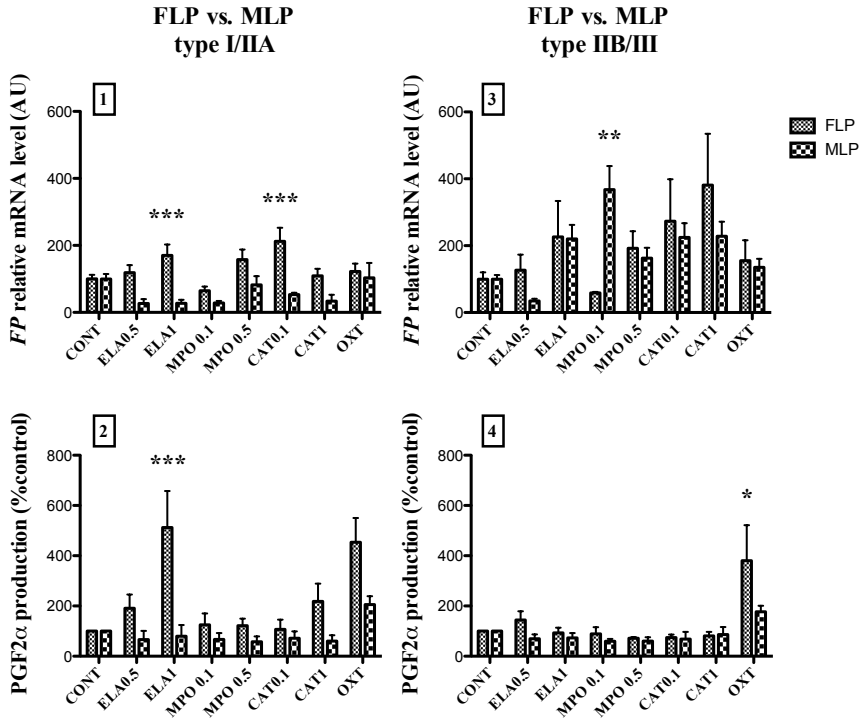

Figure 21- Effect of estrous cycle phase (follicular phase *vs.* mid-luteal phase) on PGF2α receptor *(FP)* (1, 3) gene transcription or PGF2α production (2, 4) by endometrial explants incubated with different concentrations of NETs components. N=5 samples from each group-I/IIA and IIB/III of each estrous cycle phase. Bars represent mean±S.D. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). CAT- cathepsin G (0.1 or 1 μg/mL); CONT- non-treated tissues; ELA- elastase (0.5 or 1 μg/mL); FLP- follicular phase; MLP- mid-luteal phase; MPO- myeloperoxidase (0.1 or 0.5 μg/mL); OXT- oxytocin.

No differences between estrous cycles phases were noted in *PTGS2 or AKR1C3* genes transcription after incubation ($P > 0.05$; Fig. 22.1; 22.2).

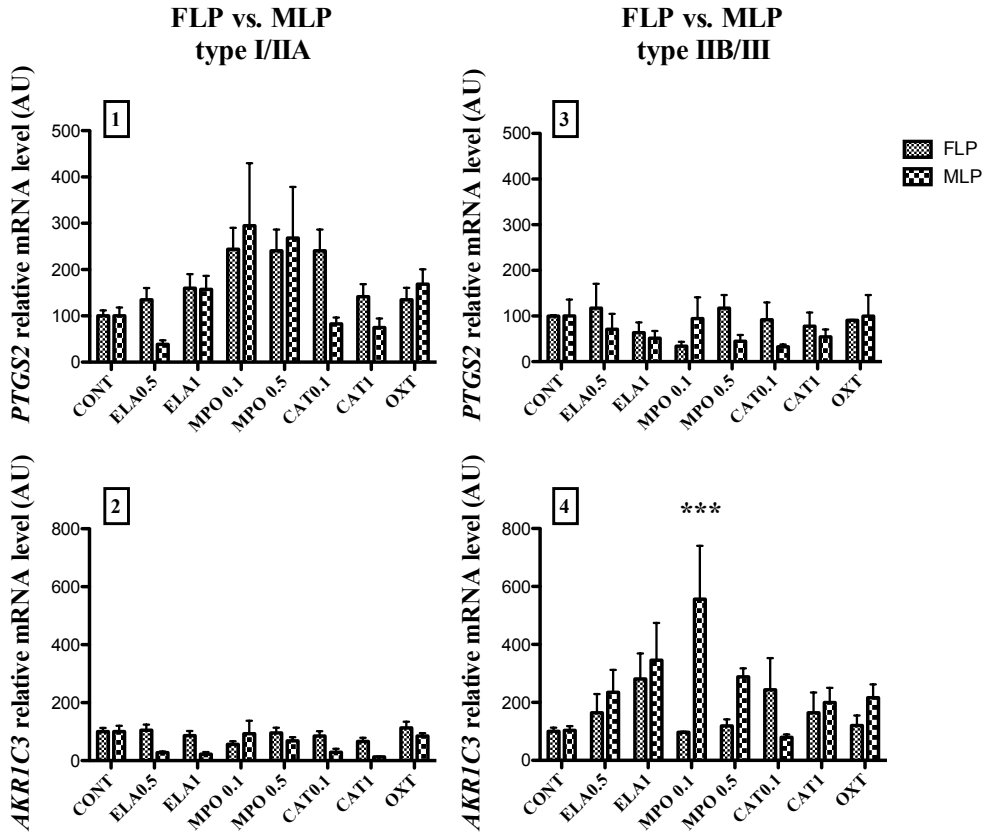

Figure 22- Effect of estrous cycle phase (follicular phase *vs.* mid-luteal phase) on prostaglandin-endoperoxide synthase 2 *(PTGS2*) (1, 3) or PGF2α synthase (*AKR1C3*) (2, 4) genes transcription by endometrial explants incubated with different concentrations of NETs components. N=5 samples from each group- I/IIA and IIB/III of each estrous cycle phase. Bars represent mean±S.D. Asterisk indicate significant differences (*** $P < 0.001$). CAT- cathepsin G (0.1 or 1 µg/mL); CONT- non-treated tissues; ELA- elastase (0.5 or 1 µg/mL); FLP- follicular phase; MLP- mid-luteal phase; MPO- myeloperoxidase (0.1 or 0.5 µg/mL); OXT- oxytocin.

4.4.4.2. Follicular phase *vs.* mid-luteal phase in type IIB/III endometria

Mid-luteal phase explants showed increased levels of *FP* ($P < 0.01$) (Fig. 21.3) and *AKR1C3* ($P < 0.001$; Fig. 22.4) genes transcription than FLP tissues, after 24h incubation with the lowest MPO dose, compared with the same treatment and endometria type during the FLP. No differences in PGF2α production (Fig. 21.4) and in *PTGS2* transcripts (Fig. 22.3) were noticed between estrous cycle phases throughout the experiment.

4.5. Discussion

In our previous work, we were able to show that NETs components could have a pathogenic role in fibrosis formation on mare endometrium, as *in vitro* incubation of endometrial explants with some NETs proteases induced high COL1 production (Rebordão *et al.,* 2018). Since mare endometrium produces abundant concentrations of PG, (Szóstek *et al*., 2012) that have

been implicated in the development of fibrosis in other tissues, we have decided to investigate some mediators of PGF2α pathway, to unravel the mechanisms behind NETs proteases-induced fibrosis. Our *in vitro* results have revealed novel changes in PGF2α pathway mediators induced by NETs proteases that may predispose to mare endometrial fibrosis establishment: (i) enhanced synthesis of PGF2α; (ii) and increased *FP* gene transcription. The type of effect that prevails was influenced not only by the type of endometrium, but also by estrous cycle phases. Those different effects may be partially explained by sex steroid hormones modulated expression of PG receptors during the reproductive cycles (Blesson & Sahlin, 2014).

Endometrosis is defined as periglandular and/or stromal endometrial fibrosis characterized by large number of smooth-muscle-actin containing myofibroblasts, pronounced epithelial vimentin expression, excessive ECM accumulation, progressive cystic glandular dilation with mechanical destruction of the uterine glands and presence of glandular nests (Hoffmann *et al*., 2009). The exact etiology and pathological mechanisms leading to endometrial destruction and progressive reproductive failure have not been fully elucidated. In fact, this pathology may somewhat result from a host defense defect that impairs bacterial killing, enhances PMN recruitment and NETs release. In the lung, when extracellular concentration of free ELA released by PMN exceeds the buffering capacity provided by endogenous inhibitors, ELA starts to drive uncontrolled inflammatory processes (Hogg & Senior, 2002). Likewise, the same may occur in the uterus of mares with NETs persistence.

It was recently reported that PGF2α acting on its receptor (FP) is capable to induce fibrogenesis in several systems. Abundant PGF2α in broncho-alveolar lavage fluid of mice with induced pulmonary fibrosis stimulated the proliferation of lung fibroblasts and COL production via FP (Oga *et al*., 2009). Also, in a bleomycin-induced pulmonary fibrosis model, plasma PGF2α metabolites were associated to disease severity and prognosis (Aihara *et al*., 2013). Increased collagen type I and III via a FP receptor/protein kinase C/Rho kinase cascade was reported in myocardial fibrosis (Ding *et al*., 2012; Ding *et al*., 2014). In mice dermal sclerosis fibroblasts, alpha2-antiplasmin (2AP), a serpin elevated in fibrotic diseases, induced PGF2α synthesis that in turn promoted fibrosis (Kanno *et al.*, 2013). Also, synovial fibrosis establishment was positively associated with PGF2α levels (Bastiaansen-Jenniskens *et al.*; 2013).

PGF2α pathway likely involved in mare endometrium fibrogenesis induced by NETs persistence was up-regulated in all studied types of endometria, except in MLP I/IIA endometria. In FLP type I/IIA tissues after 24h of NETs components insult, COL1 increase

(with the highest concentration of ELA or both doses of CAT) was associated to enhanced synthesis of PGF2α or of its receptor.

In FLP type IIB/III endometria (challenged with CAT for 24h), increased COL1 production and high transcription of *FP* gene linked PGF2α pathway to fibrosis development. Moreover, no changes in COL1 production in this same group of explants incubated with the lowest concentration of MPO, together with low *FP* gene transcription, were in agreement with the reduced COL deposition in other tissues, like in lungs (Oga *et al.,* 2009).

In contrast, in MLP type I/IIA mare endometrium, PGF2α pathway does not seem to be involved in mare endometrium fibrogenesis induced by NETs persistence. Other vias besides PGF2α pathway may be involved, since high COL1 production, induced by ELA and CAT in this type of endometrial tissues, was linked to low *AKR1C3* or *FP* gene expression. Nevertheless, in MLP type IIB/III endometrium activation of PGF2α pathway appears to be essential for fibrogenesis. All NETs proteins that induced COL1 production showed high relative transcription of *FP* or *AKR1C3* genes, except with the lowest concentration of ELA.

In this study, we investigated whether NETs effect on PGF2α pathway in mare endometrium could be dependent on the presence of endometrial fibrotic lesions. Endometrial category appears to influence the PG secretion, as enhanced *PTGS2* mRNA transcription was observed in I/IIA groups, throughout the incubation period. Although in mare endometrium no differences between endometrial categories were found in *PTGS2* mRNA transcription during the follicular and mid-luteal phases (Szóstek *et al.,* 2012), our results are in agreement with previous studies in lung fibroblasts. Diminished ability of lung fibrotic cells upregulated *PTGS2* mRNA level and fibrogenesis capacity (Keerthisingam *et al.,* 2001). Besides, increased PGF2α production after ELA treatment was only noted in I/IIA groups in FLP. A former study in which the highest concentration of PGF2α was found in healthy endometria of the mare during the follicular phase, with decreased PGF2α production in categories II and III endometria in both follicular and mid-luteal phases (Szóstek *et al.,* 2012) agrees with the present work. In contrast, type IIB/III endometria were able to respond to NETs proteases with higher *AKRC1* gene transcription, regardless of estrous cycle phase. Also, in the mid luteal phase, the type of endometrium seems to influence the levels of *FP* transcription response to NETs. In MLP type IIB/III explants higher *FP* transcripts were noted throughout the experimental period.

The putative endocrine influence on endometrial response to NETs proteases was addressed through the assessment of PGF2α mediators in tissues during FLP and MLP. In our study, increased levels of PGF2α synthesis (after ELA and CAT treatment) were only detected in FLP endometria. Such differences may be hormone-dependent and mediated by tissue

specific catabolic or anabolic enzymes involved in PG synthesis. Previous studies on steroid regulation of PG synthesis support our hypothesis. In the mare endometrium after *in vitro* exposure to estrogen (E2), a dose-dependent stimulation of PGF2α production was observed, while exposure of tissues to P4 failed to alter PGF2α secretion (Vernon *et al.,* 1981). Likewise, explants collected from mares during the follicular phase exhibited a higher basal concentration of PGF2α secretion than explants collected during the luteal phase after 24h and 72h incubation (Nash et al., 2008). Also, in equine endometrial epithelial cells, E2 appears to be a strong stimulator of PG secretion, increasing production of both PGE2 and PGF2α (Szóstek *et al*., 2014). In fact, a high level of PGF2α was found during the follicular phase in equine endometrium (Szóstek *et al*., 2014). Taken together, these effects suggest that in face of a stimulus, a higher production of both PG will occur in FLP endometria, rather than in MLP tissues, since endometrial PG synthesis mechanisms are activated. In the mare, following the entrance of bacteria or semen into the uterus, recruitment of PMNs triggers the release of PGF2α that induces uterine contractions (Woodward & Troedsson, 2013). Although this is a crucial mechanism for clearance of the uterine fluid produced by an initial inflammatory response (Katila, 1995), we postulate that sustained high levels of PGF2α due to the persistence of NETs-proteases may trigger endometrial fibrosis formation. Indeed, in mares predisposed to develop persistent endometritis (susceptible mares) have an increased number of PMN in the endometrium after breeding when compared with resistant mares (Woodward *et al*., 2013). Also, increased presence of PGF2α in susceptible mares might be due to the sustained inflammatory uterine environment in these mares (Cadario *et al*., 1999). Indeed, PGF2α has been proposed as suitable marker of uterine inflammation during mating-induced endometritis *in vivo* in the horse (Nash *et al*., 2010). Since in the mare target tissues seems to be exposed to higher concentrations of PGF2α for longer periods (Shrestha *et al.,* 2012) and an endometrial PGF2α auto-amplification system seems to exist, in which PGF2α can stimulate its own production (Kozai *et al*., 2016), this may contribute to sustained high levels of PGF2α in the uterus. So, we propose that in the presence of an insult in mare endometrium, like NETs persistence, it will induce a raise in PGF2α, strengthening the pro-fibrotic effect that will prevail.

In the present work, estrous cycle influence was not noted in *FP* gene transcription. Previous studies have suggested that sex steroid hormones that regulate the reproductive cycles, modulate the expression of PG receptors like PGE2 receptors (EPs) and FP (Blesson & Sahlin, 2014). In the uterus of ovariectomized rat, *FP* mRNA expression was decreased by estradiol (E2) treatment (Blesson & Sahlin, 2014). Nevertheless, in the mare, *FP* transcripts do not seem to be influenced by the endocrine cyclic change, since *FP* gene transcription was

similar at day 7 (MLP), 14 (late-LP) and 21 (estrus) of estrous cycle (Ruijter-Villani *et al.* 2014), although other studies have referred that the highest *FP* transcripts were observed in late luteal phase (Atli *et al*., 2010; Kozai *et al*., 2016). As in physiologic conditions, our results suggest that the mechanisms involved in *FP* gene transcription do not seem to be estrous cycle phase-dependent, since enhanced *FP* transcripts occurred both in FLP and MLP endometrial tissues under the action of NETs proteases.

Although our results suggest that PGF2α may be involved in NETs protease-induced COL1 formation in mare endometrium, as fibrosis is an extremely complex pathologic process other vias, besides the PGF2α pathway, such as PGE2 pathway and pro-fibrotic cytokines may be involved. Indeed, it has been questioned if PGF2α stimulates fibrogenesis through independent TGF-β1 pathways (Oga *et al.*, 2009; Olman, 2009; Ding *et al*., 2012; Bastiaansen-Jenniskens *et al*., 2013) or not (Kanno *et al*., 2013).

In conclusion, our results indicate that FLP endometrium seems to be less protected from pro-fibrotic mediators since it is at this stage that we have observed both high PGF2α production and *FP* gene transcription under NETs proteases action, both associated with fibrogenesis. Transcription of *FP* gene does not appear to be hormone-dependent, although its transcription seems to be dependent of endometrial category in mid luteal phase shaping fibrosis outcome. Although other pro-fibrotic cytokines may be involved, injury induced-changes on PGF2α mediators by NETs components may instigate this via to become an additional pathway with key roles in mare endometrial fibrogenesis. Besides, endocrine microenvironment and healthy or pathological conditions of endometrial tissues play an important role in fibrogenesis. In addition, further studies are needed to elucidate if putative fibrogenic PGF2α may act *in vivo* in mare endometrium, through cytokine dependent or independent mechanisms.

5. Impairment of anti-fibrotic PGE2 pathway might influence neutrophil extracellular traps-induced fibrosis in mare endometrium

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5.1. Abstract

In spite of being long recognized as a major cause of infertility in the mare, the pathogenesis of chronic endometrium fibrosis (endometrosis) is not completely understood. Prostaglandin E_2 (PGE_2) has several biological contradictory effects in many organs, such as pro- or anti-inflammatory actions, depending on the activation of its respective four subtype prostanoid receptors. By signaling through its receptors EP2 and EP4, PGE_2 mediates both anti-inflammatory and anti-fibrotic actions. We have shown that when the *in vitro* contact of neutrophil extracellular traps (NETs) proteases with mare endometrium persists, it may favor mare endometrial collagen type I production and stimulate fibrogenesis. Therefore, the objective of this study was to investigate the involvement of PGE_2 pathway in collagen deposition on mare endometrium, challenged *in vitro* with NETs proteases. Mare endometria (Kenney and Doig categories I/IIA and IIB/III), obtained in the follicular (FLP) and mid-luteal (MLP) phases, were incubated for 24h with NETs components (elastase, cathepsin-G, myeloperoxidase or oxytocin, as positive control). Expression of PGE2 protein and transcription of its synthase (*PTGES)* and receptors (*EP2*, *EP4*) genes were evaluated. Impaired PGE2 production and low *EP2* transcripts were noted. Endometrial category and estrous cycle phases influenced collagen fibrogenic output. Impairment of PGE2 pathway might play a role in FLP (category IIB/III) and MLP (I/IIA) endometrial fibrosis, due to the suppression of its anti-fibrotic capacity. In conclusion, ovarian steroid endogenous priming of the endometrium might differently modulate the anti-fibrotic PGE2 pathway in healthy or pathological tissues with opposing fibrosis outcome after NETs proteases action.

Keywords: Neutrophil extracellular traps, fibrosis, endometrium, endometrosis, estrous cycle, mare, PGE2; PGE2 receptors, endometrial pathology

5.2. Introduction

Endometrosis is a chronic, degenerative condition that affects mare endometrium. It is characterized by excessive deposition of extracellular-matrix (ECM), like type I (COL1) collagen and fibronectin, around the endometrial glands and stroma that leads to the destruction of tissue architecture and impairment of endometrium function (Kenney, 1978; Kenney & Doig, 1986; Hoffmann *et al.,* 2009; Costa *et al.,* 2015). It has been referred that persistent endometritis, as a consequence of different insults associated with breeding, foaling and veterinary interventions, can be involved in chronic degenerative changes of mare endometrium and may be related with fibrosis formation (Kenney, 1978; Ricketts & Troeddsson 2007; LeBlanc & Causey, 2009; Hoffmann *et al*., 2009).

Inflammation seems to be linked to fibrosis in a paracrine way, in which injured and inflammatory cells secrete pro-fibrotic chemokines, cytokines and growth factors that act on resident cells, like fibroblasts, and ultimately trigger fibrogenesis (Liu Y, 2011; Zeisberg & Kalluri, 2013). Prostaglandin E2 (PGE2) seems to prevent the pro-fibrotic transforming growth factor β1 (TGF-β1) to induce fibroblast differentiation to myofibroblast, thereby playing a protective role against fibrosis (Bozyk & Moore; 2011). In fibrotic lung, impaired PGE2 production and PGE2 signaling have been described (Bozyk & Moore; 2011). This eicosanoid has several biological effects in many organs, such as pro- and anti-inflammatory actions, which depend on the activation of its four subtypes prostanoid receptors (EP1, EP2, EP3 or EP4) (Kalinski, 2012). The anti-fibrotic action of this hormone is linked to either EP2 or EP4 signaling, although the subtype of receptor that is involved on this action seems to be tissue-dependent (Huang *et al.,* 2010; Ishiwata, *et al.,* 2013; Kach *et al.,* 2014; Wei *et al.,* 2014; Wang *et al*., 2017). PGE2 is a metabolite of arachidonic acid formed via the prostaglandin-endoperoxide synthase 1 (PTGS1) and /or PTGS2 enzymatic pathway. The enzyme PTGS2 is considered to be rate-limiting for PG production. Three types of PTGE synthases (PTGES) controlling PTGE2 production in cells have been identified. Two are membrane-associated (mPTGES-1 and mPTGES-2) and the third one is cytosolic (cPTGES). Expression of mPTGES-1 is responsible for sustained production of PGE2 (Fortier *et al.,* 2008) and is induced in response to inflammation (Stichtenoth *et al.,* 2001).

Polymorphonuclear neutrophils (PMN) are able to kill microorganisms not only by intracellular pathways, but also by extracellular killing mechanisms, like extracellular netlike structures called neutrophil extracellular traps (NETs) (Brinkmann et *al.*, 2004; Brinkmann & Zychlinsky, 2007). Although NETs have potent antimicrobial functions and may act as a physical barrier to prevent further spread of microorganisms (Fuchs *et al*., 2007; Lögters *et*

al., 2009; Brinkmann & Zychlinski, 2012; O'Brien *et al.*, 2017), persistence of NETs has been linked to the development of several non-fibrotic and fibrotic diseases (Jorch & Kubes, 2017; Nakazawa *et al*., 2017). In addition, it has been proposed that fibroblast activation mediated by NETs components, like histones, antimicrobial peptides, proteases, and cytokines, may contribute to disease progression towards fibrosis (Chrysanthopoulou *et al*., 2014). Several proteins have been identified in NETs and the most abundant non-histone NETs proteins are elastase (ELA), cathepsin G (CAT) and myeloperoxidase (MPO) (Urban *et al*., 2009), that have been related with fibrogenesis in different organs (Voynow *et al*., 2008; Dubois *et al*., 2012; Sharoni *et al.,* 2010; Ikegame *et al*., 2010; Segel *et al*., 2011; Cantin *et al*., 2015; Pulli *et al*., 2015).

Since NETs are present in endometrial secretions obtained from mares with bacteria endometritis (Rebordão *et al*., 2014), and *in vitro* collagen production was enhanced after challenge of mare endometrial explants with some NETs proteases, we have proposed that persistence of NETs may be involved in the development of endometrial fibrosis (Rebordão *et al*., 2018). Since PG are abundantly produced in the endometrium, modulating several physiological reproductive events (Weems *et al*., 2006) and PGE2 might hinder fibrotic conditions in several organs and species (Bozyk & Moore; 2011), we hypothesized that PGE2 might play a protective role against endometrosis in the mare. In this study, the effects of NETs proteases on the putative anti-fibrotic PGE2 pathway were analyzed by evaluating the expression of PGE2 protein and its synthases and receptor genes. The influence of endometrial inflammatory/fibrotic lesions present, as well as estrous cycle phase was also studied.

5.3. Materials and methods

5.3.1. Endometrial and blood sampling and study design

During the reproductive season (May-September), ovaries, uteri and blood from cyclic mares were collected post-mortem at the abattoir. Management and slaughter of mares were performed following European welfare (EFSA, AHAW/04–027) and Portuguese (DL 98/96, Art. 18) mandates.

By macroscopic evaluation of the ovaries (presence of a growing follicle or *corpus luteum*) uteri were identified as fitting to the follicular phase (FLP; n=10) or to the mid luteal phase (MLP; n=10). (Roberto da Costa *et al.,* 2008). Uterine horns were opened and signs of endometritis, like increased mucus production and altered color of surface epithelium were criteria to discard those genitalia. Also, uterine swabs were collected for bacteria isolation and

cytology evaluation (section 5.3.2.). None of the uteri collected were considered to be undergoing active inflammation. Immediately after uteri collection, endometrial pieces were obtained and placed in: (i) 4% buffered formaldehyde, for histopathological examination and further endometrial classification (section 5.3.2.) (ii) chilled (4ºC) Dulbecco's modified Eagle's medium (DMEM) and F-12 Ham medium (D/F medium; 1:1 (v/v); D-8900; Sigma) supplemented with antibiotics (100 mg/mL streptomycin, 100 IU/mL penicillin and 2mg/mL amphotericin- A2942; Sigma) for explant culture assays (section 5.3.3); or (iii) RNAlater (AM7020; Ambion, Applied Biosystems, Foster, CA, USA), for further mRNA (section 5.3.5) assays. Blood samples were collected for further progesterone (P4) determination in plasma, to confirm mares´ estrous cycle phases (section 5.3.6). All samples were transported on ice to the laboratory within 1h.

For *in vitro* culture studies (section 5.3.3), concentrations of ELA, MPO or CAT were chosen based in previous *in vitro* assays (Glusa & Adam, 2001; Boudjeltia *et al.*, 2004; Voynow *et al.,* 2008) and on qualitative and quantitative protein composition of NETs (Urban *et al.,* 2009; Dubois *et al.,* 2012). Besides, a preliminary dose-response trial was done to determine the concentrations of each protease that should be used. Endometrial explants were challenged with three concentrations of each protein: MPO (0.1, 0.5 or 1 μg/mL), CAT (0.1, 1 or 2.5 μg/mL) or ELA (0.1, 0.5 or 1 μg/mL) and TGF-β1 production (as a putative fibrotic marker) was assessed. Two of those tested concentrations of each protease were chosen, since the highest dose of MPO and CAT did not induce a further increase in TGF-β1 production, and the lowest concentration of ELA did not provoke a high secretion of TGF-β1, either (data not shown). In the present work, the concentrations of NETs proteases used were within the range of their levels referred in physiologic and inflammatory conditions in tissues and species other than the mare endometrium (Glusa & Adam, 2001; Boudjeltia *et al.*, 2004; Voynow *et al.,* 2008). In addition, MPO concentrations used were similar to those found in mare uterine lumen (Parrilla-Hernandez *et al.,* 2014).

This same experimental design and endometrial samples were used in our previous work that showed enhanced collagen type I production by these tissues when challenged with NETs proteases (Rebordão *et al.*, 2018). Fig. 23 summarizes COL1 protein increase that resulted after stimulation of type I/IIA or IIA/III follicular phase or mid-luteal phase endometrial explants by the different concentrations of ELA, MPO and CAT.

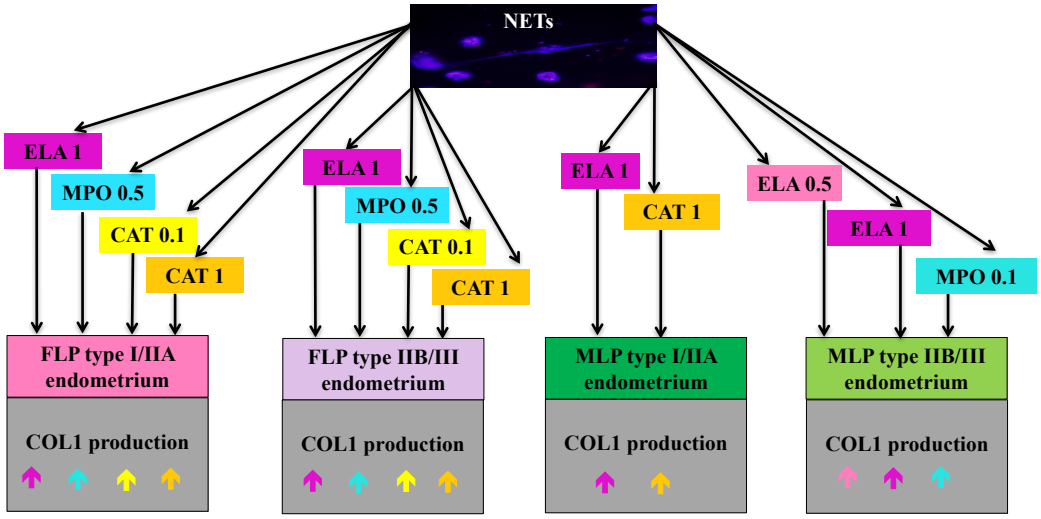

Figure 23- Summary of collagen type I protein data, in endometrial explants after incubation with different concentrations of NETs components, relative to their own negative controls (non-treated tissues). N=5 samples from each group- I/IIA and IIB/III, for each estrous cycle phase. CAT- cathepsin G (0.1 or 1 µg/mL); COL1- collagen type I; ELA- elastase (0.5 or 1 µg/mL); FLP- follicular phase; MLP- mid-luteal phase; MPO- myeloperoxidase (0.1 or 0.5 µg/mL); OXT- oxytocin.

5.3.2. Cytological and histopathological analysis of mare endometrium

Light microscopic evaluation of Diff-Quick stained endometrial smears collected with sterile swabs was used to discard the presence of inflammation and/or bacteria. Presence of acute inflammation was considered if more than two neutrophils per four microscopic fields (Mag=400X) were counted (Riddle *et al.*, 2007).

Histopathological analysis was performed on formaldehyde fixed endometrial slices (5 µm) stained with hematoxylin (05-06014E; Bio-Optica) and eosin (HT1103128; Sigma-Aldrich), (Leica DM500). Endometria evaluation were assessed to establish whether the endometrium had undergone pathological degenerative changes (Kenney & Doig 1986). Endometria were classified as belonging to category I, IIA, IIB or III, according to the degree of inflammation and/or fibrosis (Kenney & Doig 1986). Endometrial samples were then gathered into two groups for further studies. Categories I and IIA tissues were included in I/IIA group, while IIB/III group enclosed categories IIB and III endometria.

5.3.3. *In vitro* culture studies

Endometrial tissue culture was performed as previous reported (Rebordão *et al.*, 2018). Briefly, strips of endometrium (n=5 samples from each group- I/IIA and IIB/III from FLP or MLP) were cut in small pieces, slightly dried, weighed (20-30mg) and placed into each well of a sterile 24 well cell culture plate, with 1ml of DME/F-12 Ham Medium (D-8900; Sigma) containing 0.1% bovine serum albumin (735078; Roche Diagnostics, Mannheim, Germany),

streptomycin (100 mg/mL), penicillin (100 IU/mL) and amphotericin (2mg/mL). After 1h of pre-incubation, at 37ºC in a humidified atmosphere (5% CO2, 95% air) with gentle shaking, medium was changed and endometrial explants were cultured, under the same conditions, for further 24h with: (i) medium – control; or (ii) elastase (A6959, AppliChem GmbH; ELA; 0.5, 1µg/mL); (iii) cathepsin G (A6942, AppliChem GmbH; CAT; 0.1, 1µg/mL); (iv) myeloperoxidase (A6969, AppliChem GmbH; MPO; 0.1, 0.5µg/mL); or (v) oxytocin (OXT) as positive control (10^{-7} M; Nash *et al*., 2008). Each treatment was performed in quadruplicate. After, culture medium was used for explant metabolic viability assessment, using alamarBlue® or kept at -80ºC with a 1% prostaglandin stabilizer (0.3 M ethylenediaminetetraacetic acid (EDTA) and 1% aspirin, No. A2093; Sigma) (Szóstek *et al*., 2014) for further prostaglandins analysis. Endometrial explants were maintained in cryotubes with RNAlater® at -80ºC, for gene transcription studies.

5.3.4. Endometrial tissues viability assay

AlamarBlue®(AB) (DAL1100; ThermoFisher Scientific) was used to assess endometrial explants metabolic viability, as described (Carranza-Torres *et al*., 2015). Briefly, 10% of AB was added to fresh (non-incubated), to 24h incubated explants treated with NETs proteases or to not treated (negative control), as well as to culture medium. They were further incubated for 4 h in the same conditions described in 5.3.3. From each of those samples, 100 μL of medium was harvested and fluorescence values analyzed under a fluorometer microplate reader (Synergy H1 Hybrid Reader, BioTek; Gene 5 software) at 560 nm excitation/590 nm emission wavelengths. Viability of endometrial explants was obtained by calculating the percentage (%) of AB reduction per cell (Carranza-Torres *et al.*, 2015).

In addition, since $PGF_{2\alpha}$ response of non-treated and OXT-treated endometrial explants suggests that the endometrial explants contain functional cells (Nash *et al.,* 2008), $PGF_{2\alpha}$ out-put was used to assess functional capacity of incubated tissues (Nash *et al.,* 2008). In addition, PGE2 secretion response of non-treated and OXT-treated explants was also used as its functional capacity, as described in mare endometrial cells (Szóstek *et al.,* 2014).

5.3.5. Gene transcription study

Real time PCR studies of endometrial explants were performed as previously described (Rebordão *et al.,* 2017a). Total RNA was isolated with Total RNA Extraction and Purification kit (28704; Qiagen, Hilden, Germany), with a DNA-digestion step included (RNase-free DNase Set; 50979254; Qiagen), according to the manufacturer instructions. Its quality was assessed by visualization of *28S* and *18S* rRNA bands by electrophoresis on 1,5% agarose gel,

after RNA quantification with Nanodrop system (ND200C; Fisher Scientific, Hampton, PA, USA). Using a Reverse Transcriptase Superscript III enzyme (18080093; Invitrogen, GIBCO BRL, Carlsbad, CA, USA) from 1 mg total RNA in a 20 µL reaction volume using oligo(dT) primer (27-7858-01; GE Healthcare, Buckinghamshire, UK) cDNA was generated. The sequences for equine microsomal prostaglandin E synthase (*PTGES*), PGE2 receptor 2 (*EP2*), PGE2 receptor 4 (*EP4*) and ribosomal protein L32 (*RPL32*) primers were design using program Primer-3 (Untergasser *et al.,* 2012) and Primer Premier software (Premier Biosoft Interpairs, Palo Alto, CA, USA), which are shown in table 6. In a preliminary study *RPL32* has been found to be the most stable internal control gene and was used as reference gene (Rebordão *et al.,* 2018). Transcription of mRNA of both target and reference genes, was performed simultaneously in duplicate 13 µL reaction volume (3.5 µL water, 1 µL cDNA; 1µL forward primer, 1 µL reverse primer and 6.5 µL Power SYBER Green Master Mix-4367659; Applied Biosystems), in StepOnePlus™ Real-Time PCR System (Applied Biosystems, Warrington, UK). Thermocycler program was run for all genes as follows: initial denaturation (10 min at 95ºC), followed by 40 cycles of denaturation (15 s at 95ºC) and annealing (1 min at 60ºC), followed by a dissociation step (15 s at 95ºC, 30 s at 60ºC and 15 s at 95ºC). An electrophoresis on 2,5% agarose gel was run to confirm the specificity of the product. Quantification of relative mRNA data was done by real-time PCR miner algorithm (Zhao & Fernald 2005). Relative mRNA transcription of control samples was compared with treated explants data.

Table 6- Primer sequences used for *EP2*, *EP4* and *PTGES* real time PCR analysis of mare endometrial explants.

Gene (Acession number)	Sequence 5´- 3	Amplicon (base pairs)
EP2 (NM_001127352.1)	Forward: TGACCATCACCTTCGCCG	179
	Reverse: GACCGCAGCACTCTTAGCACA	
EP4 (XM_001499068.3)	Forward: CTCCCTGGTGGTGCTCATCT	219
	Reverse: CGGCAGAAGAGGCATTTGAT	
PTGES (NM_001081935.1)	Forward: CACGCTGCTGGTCATCAAGA	127
	Reverse: GGTCGTCCCGGTGAAACTG	
RPL32 (XM_001492042.6)	Forward: AGCCATCTACTCGGCGTCA	144
	Reverse: GTCAATGCCTCTGGGTTTCC	

EP2- prostaglandin E receptor 2; *EP4*- prostaglandin E receptor 4; *PTGES*-microsomal prostaglandin E synthase1; *RPL32*- ribosomal protein L32.

5.3.6. Progesterone and prostaglandins analysis

The concentrations of P_4 in plasma were determined by solid-phase Radioimmunoassay (RIA), without extraction, as previously described (Rebordão *et al.,* 2017a). Using a

commercial kit (Coat-A-Count; Diagnostic Product Corporation, Los Angeles, CA, USA), duplicate plasma P4 samples were measured under a Wallac (wizard 1470) counter, in a single assay. The limit of detection of the assay was 0.02 ng/mL and the intra-assay coefficient of variation for all samples was 3.4%. To help identify estrous cycle phase of the mares, plasma P4 concentrations were used. Were considered to be in the MLP all mares with plasma P4 values >6ng/mL, and in the FLP, mares with P4 concentrations <1ng/mL (Roberto da Costa *et al.,* 2007).

Prostaglandin $F_{2\alpha}$ in culture medium as a means to assess functional capacity of incubated tissues (Nash *et al.,* 2008) was measured by direct enzyme immunoassay (EIA), as previously described (Szóstek *et al*., 2014). Standard curve for $PGF_{2\alpha}$ ranged from 0.19ng/mL to 50ng/mL and the intra- and inter-assay coefficients of variation were on average 8.5% and 10.7%, respectively.

The concentration of PGE2 in conditioned culture medium of each sample was measured in duplicate using Prostaglandin E_2 EIA kit (PGE high sensitivity ELISA kit; Catalog nº ADI-931-001; Enzo Life Sciences), according to the manufacturer instructions. The detection limit of PGE_2 was 8.26 pg/mL. Standard curve for PGE_2 ranged from 0.039 pg/mL to 100 pg/mL, and the concentration at 50% binding (ED50) was 6.25 pg/mL. The intra- and inter-assay CV were 6.1% and 12.8%, respectively. Hormone concentrations in culture media were normalized for mg of endometrium.

5.3.7. Statistical analysis

GraphPAD PRISM (Version 5.00, GraphPad Software, San Diego, CA, USA) was used to analyze data. Statistical tests included Student's *t*-test for PGF2α or PGE2 secretion data of non-treated and oxytocin-treated endometrial explants data. One-way analysis of variance (ANOVA) followed by Dunnett *post-hoc* test was used to compare the results of endometrial explants viability and data regarding relative mRNA transcription and prostaglandin production of control and treated explants. Group of endometrium categories or estrous cycle phases influence was analyzed by Two-way ANOVA, followed by Bonferroni multiple comparison test among means. Results were expressed as mean ± S.D. A *P* value less than 0.05 was considered statistically significant.

5.4. Results

5.4.1. Viability of endometrial explants

Endometrial explants persisted viable after 24h incubation, since no differences were noted in % of AB reduction between fresh tissues (non-incubated; 98.1± 1.2%) and incubated control (non-treated) explants (94.94± 3.9%) ($P > 0.05$). Similar results were found in the % of difference in AB reduction between explants subjected to different concentrations of ELA (0.5 µg/mL= 94.62% or 1 µg/mL= 95.05%±7.5), MPO (0.1 µg/mL = 97.79%±14.7 or 0.5 µg/mL= 97.26%±12.3) or CAT (0.1 µg/mL= 95.52%±7.1 or 1 µg/mL = 98.7%±10.6) and non-treated control tissues after 24h incubation ($P > 0.05$). In addition, PGF2α and PGE2 secretion by endometrial explants after incubation with OXT (PGF2α: 9.882±4.6 ng/mg; PGE2: 8.6±5.7 pg/mg) was increased when compared with incubated non-treated tissues (PGF2α: 4.4±2.7 ng/mg; PGE2: 4.0±3.1pg/mg) ($P < 0.05$), which confirms that the endometrial explants contain functional endometrial cells. Thus, these results indicate that viability of endometrial explants was maintained in the first 24h of culture, since sustained high metabolic activity and enhanced prostaglandin secretion were observed during this time period.

5.4.2. NETs proteases on PGE2 pathway in mare endometrium

Since PGE_2 is known to be an anti-fibrotic mediator exerting this effect mainly via two types of prostanoid receptors, we have investigated if NETs proteins could be related to PGE_2 pathway, by studying PGE2 production and gene expression of *PTGES* and its *EP2* and *EP4* receptors.

5.4.2.1. Follicular phase type I/IIA endometrium explants

In type I/IIA mare endometrial explants obtained from FLP, no effects were noted in *PTGES* transcripts for any of the treatments (Fig. 24.1). However, after incubation with the lowest concentration of ELA, an up-regulation of *EP2* gene transcription was noted ($P < 0.05$; Fig. 24.2). Moreover, increased *EP4* gene transcription resulted after incubation of this same type of tissue with the highest concentrations of ELA ($P < 0.01$) or CAT ($P < 0.05$) (Fig. 24.3). Although PGE_2 production increased with the positive control OXT ($P < 0.01$), no differences were found in the production of this hormone with any of NETs proteins under study (Fig. 24.4).

5.4.2.2. Follicular phase type IIB/III endometrium explants

In FLP type IIB/III mare endometrium explants, *PTGES* transcripts did not change with any of NETs proteases treatments, but increased with OXT ($P < 0.01$; Fig. 24.5). In respect to *EP2* gene, incubation with the highest concentration of ELA, lowered its gene transcription ($P < 0.05$; Fig. 24.6). However, an up-regulation of *EP4* transcripts was observed with both concentrations of ELA ($P < 0.05$), MPO (0.5 µg/mL, $P < 0.05$) and CAT (0.1 µg/mL, $P < 0.05$) (Fig. 24.7). Impaired PGE2 production was observed, throughout the whole experimental period (Fig. 24.8), regardless of NETs proteins, except with OXT that increased PGE2 synthesis ($P < 0.05$).

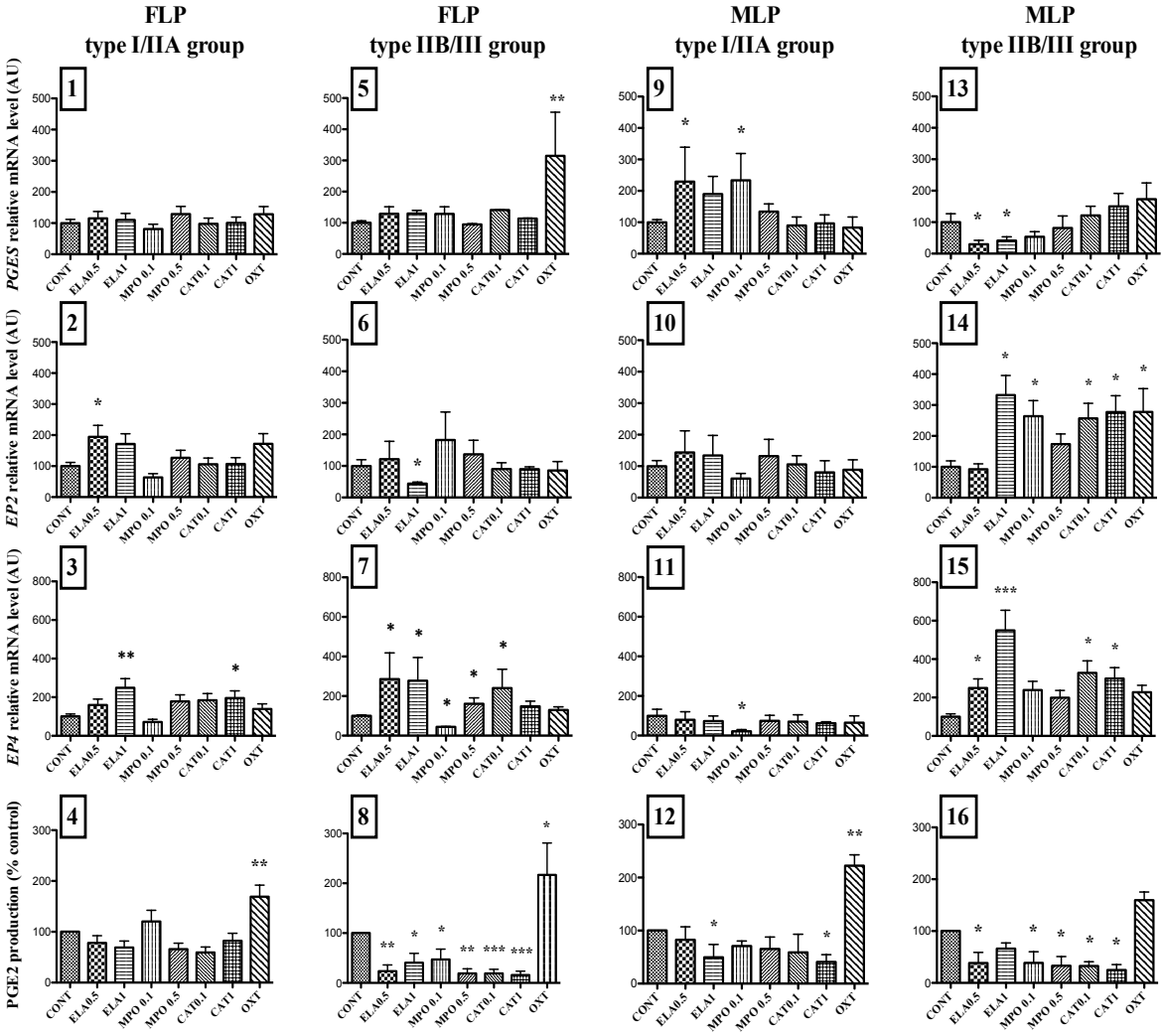

Figure 24- Relative transcription of PGE2 synthase *(PGES)* (1, 5, 9, 13), PGE2 receptors 2 *(EP2*) (2, 6, 10, 14), or 4 *(EP4)* (3, 7, 11, 15) genes or PGE2 production (4, 8, 12, 16), by endometrial explants after incubation with different concentrations of NETs components. N=5 samples from each group- I/IIA and IIB/III, for each estrous cycle phase. Bars represent mean±S.D. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). CAT- cathepsin G (0.1 or 1 µg/mL); CONT- non-treated tissues; ELA- elastase (0.5 or 1 µg/mL); FLP- follicular phase; MLP- mid-luteal phase; MPO- myeloperoxidase (0.1 or 0.5 µg/mL); OXT- oxytocin.

5.4.2.3. Mid-luteal phase type I/IIA endometrium explants

Incubation of MLP type I/IIA endometrial explants with the lowest doses of ELA and MPO, was associated with up-regulation of *PTGES* gene transcription (*P* < 0.05; Fig. 24.9). While there were no differences in *EP2* gene transcription (Fig. 24.10), decreased *EP4* transcripts were seen in explants incubated with the lowest studied concentrations of MPO (*P* < 0.05; Fig. 24.11). Likewise, the highest concentrations of ELA or CAT were associated with diminished PGE2 production (*P* < 0.05; Fig. 24.12).

5.4.2.4. Mid-luteal phase type IIB/III endometrium explants

In MLP type IIB/III endometrium decreased *PTGES* occurred after incubation with both concentrations of ELA (*P* < 0.05; Fig. 24.13). Exposure to the highest concentration of ELA, the lowest concentration of MPO and both doses of CAT, up-regulated *EP2* (*P* < 0.05; Fig. 24.14). Both tested doses of ELA or CAT increased *EP4* genes transcription (*P* < 0.05; Fig. 24.15). Nevertheless, decreased PGE2 synthesis was seen in the presence of all NETs proteins (*P* < 0.05), except with the highest tested concentration of ELA (Fig. 24.16).

5.4.3. Endometrial category influences PGE2 pathway

The influence of endometria type groups (I/IIA *vs.* IIB/III) on the effect of NETs components in putative PGE2 pathway was evaluated. This analysis was performed comparing the same treatments in the same estrous cycle phase (FLP or MLP).

5.4.3.1. Type I/IIA *vs.* IIB/III endometrium in follicular phase

No differences between endometrial categories in the follicular phase, on PTGES, *EP2* or *EP4* transcripts and PGE2 production were detected, regardless of the type or concentrations of NETs proteases (data not shown).

5.4.3.2. Type I/IIA *vs.* IIB/III endometrium in mid-luteal phase

Up-regulation of *PTGES* transcripts was observed in type I/IIA endometria after incubation with the lowest concentrations of ELA and MPO (*P* < 0.05; Fig. 25.1). In contrast, in type IIB/III endometria, higher *EP2* (*P* < 0.05; Fig. 25.2) or *EP4* gene expression was observed when tissues were incubated, for 24h, with the highest dose of ELA (*P* < 0.001), the lowest

concentration of MPO ($P < 0.05$) and CAT ($P < 0.01$; Fig. 25.3), compared with type I/IIA group. No differences between endometria type groups were observed in PGE2 synthesis, regardless of NETs proteins tested (Fig. 25.4).

Type I/IIA vs. Type IIB/III- MLP

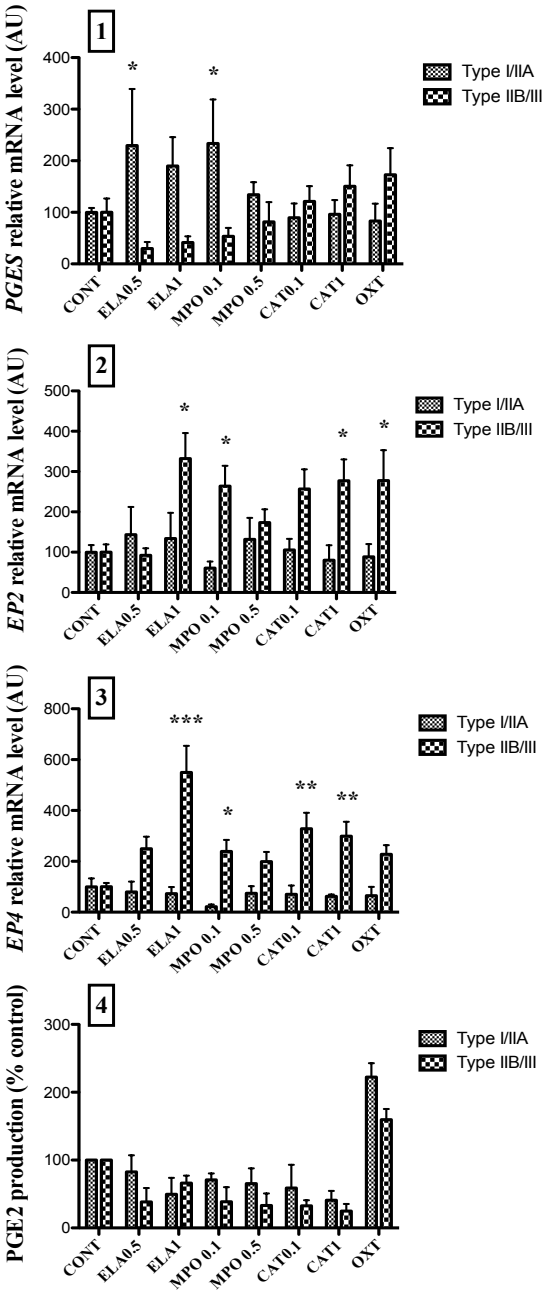

Figure 25- Effect of endometrial category (type I/IIA *vs.* type IIB/III) on PGE2 synthase *(PGES),* PGE2 receptors 2 or 4 (*EP2; EP4*) genes transcription or PGE2 production by endometrial explants incubated with different concentrations of NETs components. N=5 samples from each group- I/IIA and IIB/III in MLP. Bars represent mean±S.D. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). CAT- cathepsin G (0.1 or 1 µg/mL); CONT- non-treated tissues; ELA- elastase (0.5 or 1 µg/mL); FLP- follicular phase; MLP- mid-luteal phase; MPO- myeloperoxidase (0.1 or 0.5 µg/mL); OXT- oxytocin.

5.4.4. Estrous cycle phase influences PGE2 putative anti-fibrotic pathway

The influence of different phases of mare estrous cycle (FLP *vs.* MLP), on the effect of NETs components in PGE2 pathway was also analyzed. This evaluation was done in the same endometria types (I/IIA or IIB/III), comparing the same treatments.

5.4.4.1. Follicular phase *vs.* mid-luteal phase in type I/IIA endometria

Estrous cycle phases had no effect on *PTGES* or *EP2* mRNA gene expression, regardless of NETs proteins tested (Fig. 26.1; 26.2). However, enhanced gene transcription of *EP4* was detected in FLP endometria, treated with the highest concentrations of ELA ($P < 0.01$) and CAT ($P < 0.05$); (Fig. 26.3), compared to MLP tissues. In addition, no differences between estrous cycle phases were noted in PGE2 synthesis, regardless of NETs proteins tested (Fig. 26.4).

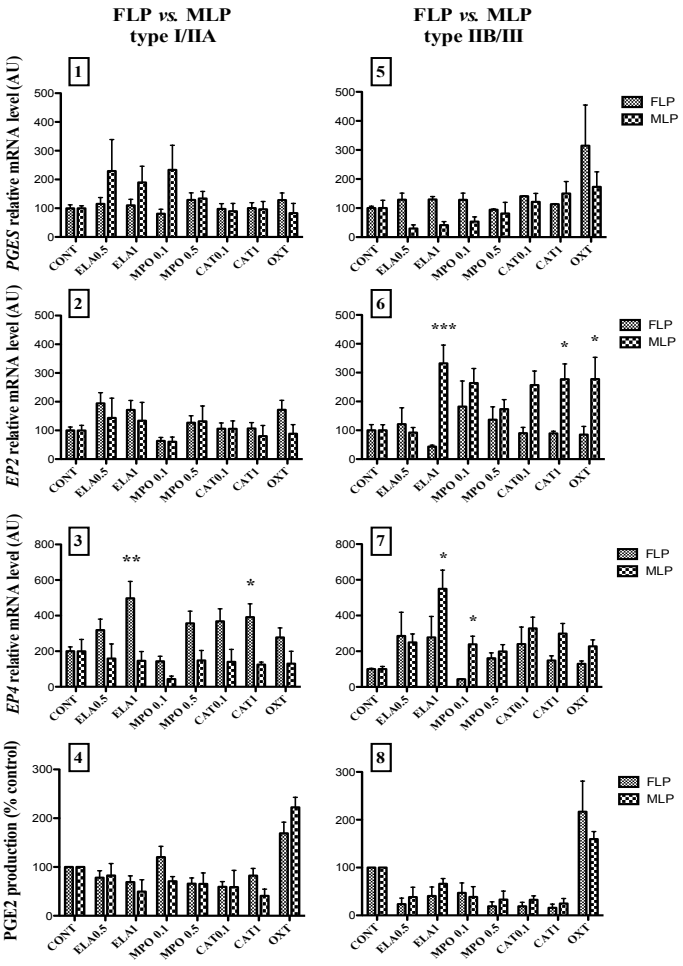

Figure 26- Effect of estrous cycle phase (follicular phase *vs.* mid-luteal phase) on PGE2 synthase (*PGES*) (1, 5), PGE2 receptors 2 (*EP2*) (2, 6), or 4 (*EP4*) (3, 7) genes transcription or PGE2 production (4, 8), by endometrial explants incubated with different concentrations of NETs components. N=5 samples from each group- I/IIA and IIB/III of each estrous cycle phase. Bars represent mean±S.D. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). CAT- cathepsin G (0.1 or 1 µg/mL); CONT- non-treated tissues; ELA- elastase (0.5 or 1 µg/mL); FLP- follicular phase; MLP- mid-luteal phase; MPO- myeloperoxidase (0.1 or 0.5 µg/mL); OXT- oxytocin.

5.4.4.2. Follicular phase *vs.* mid-luteal phase in type IIB/III endometria

When comparing FLP with MLP in tissues with severe fibrotic lesions, no difference was detected in *PTGES* gene transcription when challenged with any of NETs proteases tested (Fig. 26.5). However, in MLP, increased *EP2* transcripts were seen when endometria were challenged with the highest concentration of ELA ($P < 0.001$) and CAT ($P < 0.05$), compared to FLP (Fig. 26.6). Likewise, increased *EP4* transcripts was seen in MLP endometria treated with the highest concentration of ELA ($P < 0.05$) or with the lowest concentration of MPO with respect to FLP ($P < 0.05$; Fig. 26.7). No differences between estrous cycle phases were observed in PGE2 synthesis, regardless of NETs proteins (Fig. 26.8).

5.5. Discussion

To the best of our knowledge, this is the first report that describes the *in vitro* effect of NETs proteases on equine endometrium PGE2 production. Presence of bacteria or semen within the mare uterus stimulates *in loco* production of PGE2 and other arachidonic acid metabolites, which is followed by increased vascular permeability that enables PMN infiltration into the uterine lumen (LeBlanc & Causey, 2009). In the present study, a putative involvement of PGE2 pathway as a protective mechanism against fibrogenesis should be considered. In FLP type I/IIA endometria subjected to the lowest concentration of ELA, enhanced *EP2* gene transcription was simultaneous with no differences in COL1 production (Rebordão *et al*., 2018), suggesting that *EP2* might have an anti-fibrotic effect. Nevertheless, in endometria with severe fibrotic changes (type IIB/III), NETs proteases, mainly ELA and CAT, impaired PGE2 production in both phases of the estrous cycle. Likewise, a decrease in *in vitro* basal secretion of PGE2 in equine category III endometrium compared to category I has been referred (Szóstek *et al.,* 2012). This might be a favorable condition for endometrosis establishment in mares considered susceptible to endometritis.

Interestingly, three recent reports recognized PGE2 as an endogenous physiological inhibitor of NETs release through EP2 signaling (Marey *et al*., 2014; Shishikura *et al*., 2015; Domingo-Gonzalez *et al*., 2016). It was proposed that PMN have a universal feature that consists of NETs inhibition by PGE2 signaling, and that PGE2 can inhibit NETs production itself (Domingo-Gonzalez *et al*., 2016). Therefore, this role of PGE2 appears to be directly related to its EP2-EP4 mediated anti-inflammatory and anti-fibrotic effects and might be a mechanism of protection against chronic diseases development. In the mare, based on our data, PGE2 anti-fibrotic effect appears to be mediated by *EP2* receptor. This is in agreement with work on lung fibrosis where EP2 has been shown to be responsible for PGE2 anti-fibrotic effects (Huang *et al*., 2010; Kach *et al*., 2014; Wei *et al*., 2014). Our results suggest

that in mare endometrium, EP4 does not seem to have a protective effect against fibrosis. This is in contrast with the protective roles of endogenous PGE2-EP4 signaling against cardiac fibrosis (Ishiwata *et al*., 2013; Wang *et al.,* 2017) and renal fibrosis (Nakagawa *et al.,* 2012). Down-regulation of *EP4* gene transcription was detected in FLP type IIB/III and MLP type I/IIA groups, without any change in COL1 production (Rebordão *et al.,* 2018). Furthermore, enhanced COL1 protein (Rebordão *et al.,* 2018) was associated with up-regulation of *EP4* transcripts in FLP type I/IIA groups. Besides, *EP4* gene transcription does not appear to be estrous cycle phase-dependent, since increased *EP4* transcripts were detected in all studied endometrial types under NETs proteases action. This effect is probably related to EP4 physiological expression during the estrous cycle, since its gene expression is not affected by endocrine cyclic changes (Atli *et al*., 2010).

As increased COL1 formation in severe endometrosis (Rebordão *et al.,* 2018), and low *EP2* transcripts were only seen in FLP endometria challenged with the highest dose of ELA, this may be involved in the mechanism of fibrogenesis in this type of endometrium. Under physiological conditions, it is in the mid-luteal phase that there is an increase of *EP2* transcripts in mare endometrium (Atli *et al.,* 2010; Gebhart *et al.,* 2012). Thus, this may be the reason why decreased endometrial *EP2* gene expression was only detected in follicular phase. So, under physiological conditions, FLP endometrium may be more prone to develop fibrosis in the presence of a pro-fibrotic injury, due to a diminished capacity to synthetize this anti-fibrotic mediator. Also, in MLP endometrium with minimum or mild pathological alterations (type I/IIA), PGE2 pathway might trigger fibrogenesis, due to the suppression of PGE2 anti-fibrotic action. High COL1 production induced by ELA and CAT in these types of tissues (Rebordão *et al.,* 2018) was associated with impaired PGE2 production throughout the experimental period, without any changes in the other PGE2 pathway mediators studied. Since PGE2 might prevent fibroblast differentiation through TGF-β1 inhibition (Bozyk & Moore; 2011), we hypothesize that impaired PGE2 synthesis may provoke mare endometrial fibrogenesis.

In mare endometrium explants, stimulated with NETs proteases, a decrease in PGE2 production was observed. Exposure of human bronchial epithelial cells (HAEC) to ELA, increased PGE2 release within 10 min of incubation and peaked at 3 h, starting to decline thereafter (Perng *et al.,* 2003). It was suggested that this might represent a natural defense mechanism of the airways to regulate local inflammation (Perng *et al.,* 2003). Also in the mare, in inflamed uteri, increased secretion of PGE2 has been reported (Gajos *et al.,* 2014). Since increase of PGE2 may inhibit PMN phagocytosis and NETs release (Marey *et al.,* 2014; Shishikura *et al.,* 2015; Domingo-Gonzalez *et al.,* 2016), this might lead to persistence of

both inflammation and NETs. Nevertheless, despite an initial release of PGE2 with local anti-inflammatory properties, a later impairment in PGE2 release occurs in fibrotic lungs (Bozyk & Moore, 2011). This is in agreement with our *in vitro* data on mare endometrial explants where a fall in PGE2 was detected. In the fibrotic lung, multiple mechanisms are responsible for PGE2 loss, like soluble mediators and epigenetics (Bozyk & Moore, 2011). Injury to lung epithelial cells by pro-fibrotic mediators provokes the release of chemokine CCL2 and diminished PGE2 production, triggering fibrogenesis (Moore *et al.,* 2003).

In the mare, the intrauterine administration of PGE2 causes an increase in uterine tone and contractility (Troedsson *et al.,* 1995; Volkmann *et al*., 1995; Gastal *et al.,*1998). Based on our *in vitro* results, with impaired PGE2 release by endometrial explants challenged with NETs proteases, we might speculate that *in vivo* NETs persistence in mare uterine lumen may lead to impaired PGE2 production. This possibly contributes for impaired physical clearance, which is the main factor involved in susceptibility to persistent endometritis (LeBlanc & Causey, 2009). However, this hypothesis needs further confirmation with *in vivo* trials.

In conclusion, our results suggest that PGE2 may have a protective role against mare endometrial fibrosis, mainly mediated by its receptor EP2 but not EP4. Once fibrosis is an extremely complex condition, other pathways rather than PG, such as pro-fibrotic cytokines should be considered.

Chapter IV- General discussion

Steroid hormones, prostaglandins and oxytocin are important hormones that dictate different physiological events in female reproductive tract, namely in the uterus. Nevertheless, some of them may have dual and antagonistic effects depending on the endometrial endocrine environment. In addition, these hormones may, in specific situations, be involved in the development of pathologies, like the development of endometrial fibrosis in the mare. In the endometrium, endocrine environmental changes modify the cross-talk between the different cells which may modulate the type of response of this tissue to the same type of stimulus. An example of this is the action of OXT. While it has a pro-luteolytic effect between days 10 and 15 after ovulation, it acts as an anti-luteolytic factor during mid-luteal phase (Rebordão *et al.*, 2016; Vanderwall *et al.*, 2016). This latter effect is the base of its use as a method to prolong luteal function and suppress estrous behavior in the mare.

In the first part of our work the aim was to evaluate if chronic OXT administration to mares in mid-luteal phase would be able to prolong luteal function by modulating PG pathways, steroid hormones and OXT receptors in the endometrium. Since we sought to compare mid-luteal phase endometrial conditions prior to luteolysis with conditions following failed luteolysis induced by OXT administration, endometrial expression studies were performed in day 10 mid-diestrus in control mares and in day 24 in prolonged CL mares. Besides, as pregnancy maintenance requires the inhibition of the luteolytic mechanism during late diestrus, this physiological state could perhaps serve as a model for the mechanism by which luteal maintenance is achieved in the absence of conception. Our work suggests that administration of exogenous OXT on days 7 to 14 after ovulation is an effective method of disrupting luteolysis to prolong CL function, as previously reported (Vanderwall *et al.,* 2007; 2012; Keith *et al.,* 2013; Vanderwall *et al.*, 2016). Administration of OXT to mares in mid luteal phase was able to block luteolysis and extend the luteal phase in 67% (4/6 mares) treated mares. Prolonged luteal function associated with chronic OXT treatment seems to be related to increased and different spatial expression of OXTR and PGR in the endometrium. The detection of increased PGR protein in luminal and glandular epithelium in the OXT-treated group differs from the results obtained in earlier studies with prolonged luteal function in mares, as in diestrus and early pregnancy, wherein PGR was not detected in luminal epithelium (Hartt *et al.,* 2005; Silva *et al.*, 2014). Also, in OXT treated mares, increased endometrial OXTR protein expression was found in stromal cells. Since an initial up-regulation of OXTR in the luminal epithelium is needed to trigger luteolysis (Ruijter-Villani *et al.,* 2015), chronic OXT administration in mares might shift cell-specific expression of OXTR from luminal epithelium to stroma, leading to luteal maintenance. The observed reduction of endometrial ESR2 in OXT-treated mares may be responsible for the maintenance

of PGR in luminal and glandular epithelium. Decreased ESR2 may attenuate the transcriptional activity of *ESR1* in the endometrium, which is responsible for down-regulation of PGR in luminal epithelium, as described in mouse uterus (Weihua *et al.*, 2000).

In the second part of this work, we postulate that some mares might fight bacteria that cause endometritis by inducing NETs release, since presence of bacteria or semen in the mare uterus induces an inflammatory state characterized by an influx of PMN to uterine lumen (LeBlanc & Causey, 2009; Cadario, 2014). It was shown that human PMN were able to release their DNA in response to infectious stimuli and form neutrophil extracellular traps (NETs) that trap and kill bacteria (Brinkman *et al.* 2004). Also, *in vitro* studies revealed that in the presence of spermatozoa, equine PMN were also capable to induce NETs (Alghamdi & Foster, 2005, Alghamdi *et al.,* 2009). Thus, we aimed to evaluated the capacity of equine PMN to produce NETs not only *in vitro* by chemical activation, or stimulated with *Streptococcus equi* subspecies *zooepidemicus* (Szoo), *Escherichia coli* (Ecoli) or *Staphylococcus capitis* (Scap) strains obtained from mares' endometritis, but also *in vivo* in endometrial mucus from mares with bacterial endometritis. In this study, we were able to show that stimulated equine PMN are able to release NETs in the presence of specific Gram-positive and Gram-negative bacteria causing uterine infection in the mare, as for other PMN species and bacteria (Brinkman *et al.* 2004). Also, this study showed that bacteria causing mare endometritis were ensnared by NETs showing a direct contact between NETs and bacteria. Besides, the expression of histones, myeloperoxidase and neutrophil elastase in *in-vivo* NETs, suggested that they may have antimicrobial activity, as proposed for other bacteria and parasites (Brinkmann *et al.*, 2004).

Recently it has been referred that whenever NETs release is not physiologically controlled, NETs persistence can either fight disease but also can trigger tissue injury and cause disease themselves (Brinkmann & Zychlinsky, 2012; Corsiero *et al.*, 2016; Nakazawa *et al.*, 2017). Thus, in the third part of this work we postulated that persistence of NETs components may trigger a harmful effect on the endometrium leading to collagen deposition and fibrosis establishment in the mare. Endometrosis is a consequence of diverse insults that result in degenerative changes of the uterine glands and surrounding stroma, characterized by periglandular arrangement of myofibroblasts and deposition of extracellular matrix (ECM) (Kenney & Doig, 1986; Hoffmann *et al.*, 2009a). The exact mechanisms behind endometrial fibrosis remains obscure. In the mare, persistent endometritis due to different injuries like microorganisms or semen, leading to chronic inflammation has been related with chronic degenerative changes of mare endometrium and related with fibrosis formation (Kenney, 1978; Kenney & Doig, 1986; Ferreira-Dias *et al.*, 1994, 1999; Ricketts & Troeddsson, 2007;

LeBlanc & Causey, 2009). Chronic endometrial inflammation/infection produces a uterine response characterized by mucus overproduction by epithelial cells, transudation of plasma proteins and an influx of immunoglobulins and PMN to uterine lumen (Cadario 2014). PMN release a number of chemical mediators, including oxygen-free radicals as they phagocytize bacteria and debris (LeBlanc & Causey, 2009). Persistence of these by-products of inflammation in uterine lumen for 48h, due to deficient uterine clearance, increases the likelihood of cell damage (LeBlanc & Causey, 2009). Also, the extracellular presence of PMN serine-proteases (like ELA, CAT and MPO) in other tissues than endometrium has been linked to an overwhelming inflammatory response and to the development of fibrosis in several human organs (Rudolph *et al.,* 2010; Cantin *et al.,* 2015; Pulli *et al.,* 2015).

Therefore, the propose was to address the *in vitro* effect of some NETs proteases (ELA, MPO or CAT) on mare's endometrial capacity to form fibrosis. In this work, increased COL1 production by endometrial tissues, characteristic of fibrosis was observed with all studied NETs proteins, although estrous cycle phase and/or endometrial category affected endometrial response to each specific NETs protease. Thus, the follicular phase, appears to be the stage at which the mare has increased risk of developing this pathology. Besides, endometrium with severe fibrotic lesions, bacterial attachment was significantly higher in the estrus phase of the cycle (Ferreira-Dias *et al.*,1994). Nevertheless, it is at this stage that open cervix and uterine contractions will contribute for uterine clearance of bacteria, inflammatory products and cellular debris (LeBlanc & Causey, 2009). However, ELA enhanced COL1 production in all endometrial groups, regardless of estrous cycle phase. Our results are in agreement with those obtained by others, in which increased collagen production was observed when lung fibroblasts were treated *in vitro* with NETs (Chrysanthopoulou *et al.*, 2014). In the mare, after experimentally induced bacterial endometritis, a temporary activation of fibrotic stromal cells was seen, although no exacerbation of endometrosis during the 2-year period was detected (Hoffmann *et al.*, 2009a). In another study with low correlation between inflammation and endometrosis grade, it was proposed that progression of the fibrotic process after a critical point becomes independent of inflammation, despite the presence of inflammation preceding endometrial fibrosis (Aresu *et al.*, 2012). However, in a recent study, mares with closed cervix had larger accumulations of fluid and higher numbers of PMNs in uterine fluid and in all mares periglandular fibrosis scores increased during the experiment (Reilas *et al.*, 2016). Although our *in vitro* data suggests that NETs involvement in chronic endometritis in the mare may act as putative endometrial fibrogenic mediators, further studies are required to confirm if in *in vivo* conditions the same might occur.

In the last two parts of this work we tried to address one of the multiple putative mechanisms involved in the extremely complex pathogenesis of mare endometrial fibrosis. In our study, increased levels of PGF2α synthesis, after ELA and CAT treatment were only detected in follicular phase type I/IIA group endometria and were associated with increased COL1 protein. Nevertheless, enhanced COL1 and increased levels of *FP* gene transcription were detected in all types of follicular phase tissues after CAT treatment. Also in MLP type IIB/III explants higher *FP* transcripts were noted throughout the experimental period. Both of those conditions may trigger fibrogenesis in mare endometrium, since increased PGF2α acting on its receptor (FP) is capable to induce fibrogenesis in several systems Oga *et al.*, 2009; Aihara *et al.*, 2013; Ding *et al.*, 2012; Kanno *et al.*, 2013; Ding *et al.*, 2014). In the mare, following the entrance of bacteria or semen into the uterus, recruitment of PMNs triggers the release of PGF2α that induces uterine contractions (Woodward & Troedsson, 2013). In mares predisposed to develop persistent endometritis (susceptible mares) have an increased number of PMN in the endometrium after breeding when compared with resistant mares (Woodward *et al.*, 2013). Also, increased presence of PGF2α in susceptible mares has been related to the sustained inflammatory uterine environment in these mares (Cadario *et al.*, 1999). Likewise, in the mare target tissues seem to be exposed to higher concentrations of PGF2α for longer periods (Shrestha *et al.* 2012), and an endometrial PGF2α auto-amplification system seems to exist, in which PGF2α can stimulate its own production (Kozai *et al.*, 2016). This may contribute to sustained high levels of PGF2α in the follicular phase. Thus, we postulate that sustained high levels of PGF2α due to NETs-proteases persistence may trigger endometrial fibrosis formation.

The hormone PGE2, signaling through EP2 and EP4, is known to be an anti-fibrotic mediator (Sokolova *et al.*, 2005), although the subtype of receptor that is involved on this action seems to be tissue-dependent (Huang *et al.*, 2010; Ishiwata, *et al.*, 2013; Kach *et al.*, 2014; Wei *et al.*, 2014; Wang *et al.*, 2016). Our results suggest that in mare endometrium, EP2 might be responsible for putative PGE2 anti-fibrotic effects. Impaired PGE2 (Vancheri *et al.*, 2000; Sokolova *et al.*, 2005; Moore *et al.*, 2000) or EP2 receptor in tissues (Huang *et al.*, 2010), prevents PGE2 from exerting its anti-fibrotic functions. In our study, impaired PGE2 synthesis were noted in the endometrium of mares with moderate to severe lesions (type IIB/III), regardless of estrous cycle phase. Since increased COL1 formation and low *EP2* transcripts were only seen in FLP endometria challenged with the highest dose of ELA, this may be involved in the mechanism of fibrogenesis in this type of endometrium. Under physiological conditions, it is in the mid-luteal phase that there is an increase of *EP2* transcripts in mare endometrium (Atli *et al.*, 2010; Gebhart *et al.*, 2012). Thus, this may be

the reason why decreased *EP2* gene expression was only detected in follicular phase endometrium. So, under physiological conditions, FLP endometria may be more prone to develop fibrosis in the presence of a pro-fibrotic injury, due to a diminished capacity to synthetize this anti-fibrotic mediator. Also, in MLP endometria with minimum or mild pathological alterations (type I/IIA), PGE2 pathway might play a key role triggering fibrogenesis, due to a deletion of PGE2 anti-fibrotic action. High COL1 production induced by ELA and CAT in these types of tissues was associated with relatively impaired PGE2 production throughout the experimental period, without any changes in the other PGE2 pathway studied mediators and linked to low *AKR1C3* or *FP* gene expression. Since PGE2 seem to prevents TGF-β1 induced differentiation of fibroblasts to myofibroblasts, thereby playing a protective role in fibroblast differentiation (Bozyk & Moore; 2011), impaired PGE2 synthesis may provoke endometrial fibrogenesis through TGF-β1 activation.

Although our results suggest that PG may be involved in NETs protease-induced COL1 formation in mare endometrium, as fibrosis is an extremely complex pathologic process, other vias besides prostaglandins pathways, such as pro-fibrotic cytokines may be involved. Moreover, further studies are needed to elucidate if putative fibrogenic PG may act through independent mechanisms.

Although being one of the main subjects of research in mare reproductive research, the complete mechanisms that lead to endometrial fibrosis and its complex etiology remains to be fully elucidated. Our *in vitro* data suggest that NETs components might act as pro-fibrotic mediators in mare endometrium, even though an association between the development of endometrosis and endometrial chronic inflammation is a controversial topic. Further *in vivo* studies are needed in order to confirm that these proteases are increased in the uterus of mares with persistent endometritis. Although increased uterine MPO concentrations in cases of acute endometritis in the mare have been reported (Parrilla-Hernandez *et al.*, 2014), there is no information in subclinical conditions nor studies on ELA and CAT. Understanding the causes and mechanisms that are involved in endometrial fibrosis development in the mare, may contribute not only to slow down the progression, but also to counteract this pathological process. Although fibrosis has been believed to be irreversible for a long time, many researchers also predicted reversibility of fibrosis because fibrosis can regress when the causative conditions are removed both in human patients and in rodent models (Seki & Brenner, 2015).

Chapter V- Conclusions

The results obtained in this work offer new knowledge not only in the cellular and molecular mechanisms involved in OXT-induced luteal maintenance in the mare, but also in possible pathways involved in the development of mare endometrial fibrosis. Data analysis regarding these two issues allow us to draw the following conclusions:

1. Administration of exogenous OXT on days 7 to 14 after ovulation is an effective method of disrupting luteolysis and prolonging luteal function in the mare. The different spatial expression of OXTR and PGR in the endometrium may be a mechanism by which chronic OXT enables CL persistence. The reduction of endometrial ESR2 may be responsible for the maintenance of PGR in luminal and glandular epithelium.
2. Equine PMN are able to release NETs in the presence of bacteria that cause mare endometritis, both *in vitro* and *in vivo*, and might be a complementary mechanism by which mares can combat endometritis.
3. Increased COL1, characteristic of fibrosis, was observed after *in vitro* incubation of endometrial explants with all NETs proteases, although endometrial response to each NETs protease depended on estrous cycle and/or endometrial category. Since elastase enhanced COL1 production, regardless of endometrial category or estrous cycle, this suggests that NETs persistence might be linked to mare endometrial fibrotic pathology.
4. Enhanced synthesis of PGF2α and/or increased *FP* gene transcription were detected in follicular phase type I/IIA group endometria and were associated with increased COL1 protein, both conditions associated to pro-fibrotic effects of PGF2α pathway.
5. Increased COL1 formation and impaired PGE2 or *EP2* transcripts observed in FLP type IIB/III endometria or in MLP endometria with minimum or mild pathological alterations (type I/IIA) challenged with the highest dose of ELA might have a key role triggering fibrogenesis, due to a deletion of PGE2 anti-fibrotic action.
6. Follicular phase endometria may be more prone to develop fibrosis when triggered by a pro-fibrotic stimulus. It was at this stage that a reported pro-fibrotic association between both high PGF2α production and low *EP2* transcripts under NETs proteases action was noted.
7. NETs components induced changes on PG mediators may instigate PGF2α or PGE2 vias to become additional pathways with key roles in mare endometrial fibrogenesis.

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